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Evaluation of epinephrine and vasopressin toxicity in human mesenchymal stem cells (hMSCs)– in vitro study

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Amirpoya Akasheh

Dedicated to my lovely wife, mother and sisters, my father for their love and support….

“The knowledge of anything, since all things have causes, is not acquired or complete unless it is known by its causes.”

Contents

1 Abstract

Purpose: Epinephrine is an effective vasoconstrictor, and it is commonly administered intra-articular during arthroscopy to control the bleeding as a local hemostatic agent. However, no study so far has investigated possible toxicities of either epinephrine or an alternative like vasopressin on human mesenchymal stem cells.

Methods: Triple-layer culture flasks have been used to seed the hMSCs; we have seeded the cells at a density of 10,000 cells/cm^2. The hMSCs were plated into 96-well plates for evaluation of mitochondrial activity or 6-well plates for evaluation of apoptosis, and all the plates contained culture medium. Cells were treated with either epinephrine or vasopressin, and the control group was untreated. Epinephrine and vasopressin were removed after forty minutes, and freshly prepared growth medium has been provided to the cells. We evaluated two apoptosis markers (CASP3 and PARP-1) and the activity of the mitochondria at one hour, twenty-four hours, and seven days.

Results: Mitochondrial activity did not significantly decrease after exposure to either epinephrine or vasopressin. In the cells exposed to epinephrine, we noted a significant increase in PARP-1 level at seven days; we noted the same increase after exposure to vasopressin at twenty-four hours and seven days. The amount of CASP3 has significantly increased only at twenty-four hours in those cells which were exposed to epinephrine.

Conclusions: These data suggest that neither epinephrine nor vasopressin influence mitochondrial activity in hMSCs in vitro. We observed an increase in apoptosis markers without a corresponding decrease in mitochondrial activity, which could indicate that both medications are toxic to hMSCs. The absence of a response of CASP3 after exposure to the vasopressin, suggest that vasopressin may be

less toxic for hMSCs. However, while epinephrine and vasopressin are effective as hemostatic agents, they may have toxic effects on hMSCs, warranting caution in their use during arthroscopy, especially if performing the kind of procedure to induce the tissue repair and regeneration (for example, meniscal-repair, microfracture, tendon-repair, OATS, ACI, etc.).

2 Introduction

2.1 Stem cells

The stem cells have the excellent capability of differentiation into various cell types depending upon the needs of an organism; thus, they are described as multipotent; differentiation is determined on the base of complex signals at different stages of development and regeneration. Stem cells are also capable of replicating and replacing a cell that is similar to themselves (self-renewal) or another type of cells, called daughter cells which will turn into stem cells too (symmetrical self-renewal); they can also become specialized, progenitor cells (asymmetrical self-renewal) (1).

Adult stem cells are multipotent and exist everywhere in the whole body; they live in the vast number of body tissues and many organs, similar to pluripotent embryonic stem cells (ESCs). Adult stem cells have a unique microenvironment (niches or lacuna) that is surrounded by neurons, blood vessels, soft tissue, and supportive cells (1). This microenvironment supports and preserves the number of stem cells with no damage to DNA or differentiation and self-renewal abilities, which depend on the demand of the tissue (2, 3). Their capability to differentiate into a vast diversity of specialized tissues means that they may have medical applications in transplantation (4), organ and limb regeneration (5), genetic disorders (6), cancer (7), and immune dysfunction (8).

There are three types of stem cells: Embryonic stem cells (ESCs), induced pluripotent stem cells (iPSCs), and tissue stem cells (adult stem cells). After the embryonic stage of life, all of the stem cells are recognized as somatic, tissue, or adult stem cells, which are named according to their origin and are usually multipotent: that is, they are capable of differentiating to a limited number of precursor cells. At this stage, the stem cells can grow and differentiate on cell culture plastics. Here, we focused on

hMSCs (human mesenchymal stem cells), which are often used in evaluations in vitro growth and differentiation. While bone marrow has the richest supply of MSCs (mesenchymal stem cells), they can also be obtained from the umbilical cord, fat tissue, and dental pulp (9).

2.2 Human MSCs

MSCs are multipotent cells that can be utilized in cell-based therapeutic strategies; these applications are primarily due to the innate potential of MSCs in regeneration and differentiation into other types of cells in the appropriate physiological environment. Human MSCs (hMSCs) are widely accepted as a good origin for stem cells, as they have proven their effectiveness in various therapeutic strategies (10). There are some limitations associated with the therapeutic applications of stem cells, such as the high rate of immune-associated complications. However, these immune reactions are reduced when hMSCs are included in therapy (11).

As discussed earlier, a variety of sources can be used as a source of hMSCs. Moreover, like ESCs, hMSCs have the capability to give rise to numerous types, such as osteoblasts, adipocytes, chondrocytes, myoblasts, and neurons (Figure 1). Also, hMSCs are able to identify and home to target tissue in response to stimuli (12).

In the early neonatal phase, MSCs reside in bone, next to the red marrow, which is surrounded in there by bone, vasculature, and neural tissue. They have several different specifications that make them amenable to laboratory applications. Under ideal and standard culture situations, they can adhere to plastic flasks and dishes used for tissue and cell culture, researchers have established the following markers found on the surface of the cell: for example, CD73-positive, CD90-positive, CD105-positive; CD11b-negative, CD14-negative, CD19-negative, CD34-negative, CD45-negative, CD79α-negative,

and HLA-DR-negative (13, and 14), and in vitro they can be induced to differentiate into mesodermal cells. They are also able to differentiate to fibroblasts, osteoblasts, chondrocytes, and adipocytes and, in some situations, to tenocytes, neuroblasts, hepatocytes, and myoblasts (13, and 14) (Figure 1).

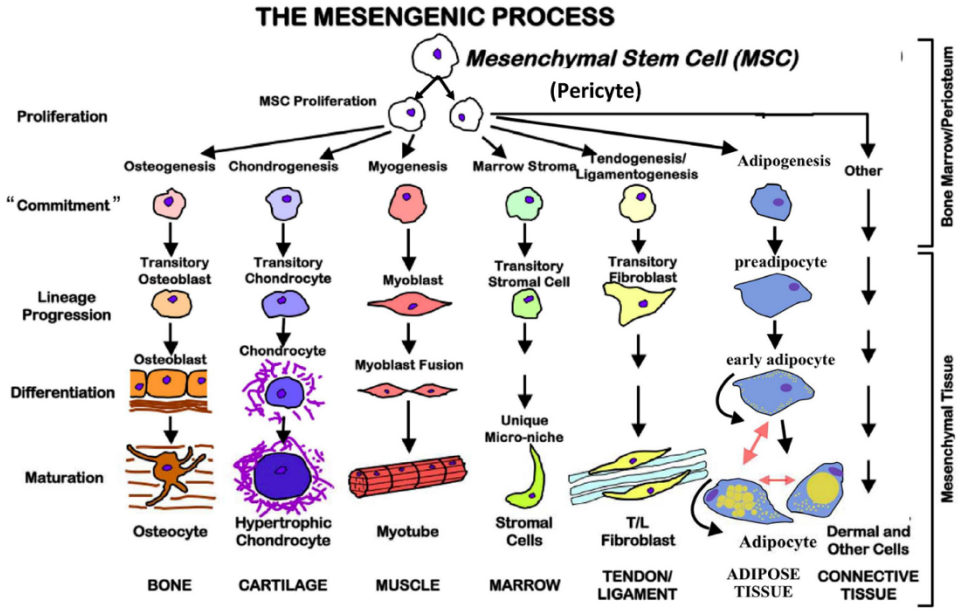

FIGURE 1. Adapted from Dimarino et al. (2013). The differentiation process in mesenchymal tissue. The MSCs are multipotent and may proliferate and differentiate in response to environmental signals. They may also be reprogrammed to another lineage. (39).

An interesting aspect of MSCs is the association of the type of focal adhesion complex and the capability of differentiation in MSCs into adipocytes or osteoclasts. Those MSCs with a few, small adhesions from fat and those that are larger (> 5 μm in length) and more numerous form bone (9). This data has exciting implications for cartilage, tendon tissue, and bone regeneration (9, 15, and 16).

2.2.1 Osteoblasts

Osteoblasts differentiate from MSCs. They produce organic materials in a cartilaginous base, forming hard tissue, and eventually encase themselves in a closed porous bony area (lacunae) before terminal differentiation into osteocytes. The Haversian systems are the end products of osteoblast activity, and under the microscope, they display the standard appearance of calcification with central canaliculi for neural fibers and blood vessels. Inside the lacunae, osteocytes are surrounded by compact bone and make connections with their neighbors with several cell projections (9).

2.2.2 Chondrocytes

Upon MSCs differentiation, chondroblasts secrete proteoglycan and collagen fibers around themselves and transform to chondrocytes, which maintain and synthesize cartilage. Inside the cartilage, chondrocytes remain in a small, closed living area and secrete chemicals for maintenance of the tissue (9).

2.2.3 Fibroblasts

Fibroblasts are the main cellular compartment of the extracellular matrix (ECM). Fibroblasts provide integrity and help the structure of connective tissues by secreting various proteins, primarily collagen fibers, fibrin, and other ground substances. Fibroblasts are the most crucial part of the tissue for adaptation because they sense and respond to the surrounding stimuli through enzymes and a complex of protein receptors (9).

2.2.4 Adipocytes

Adipocytes store lipids; they are another derivative of MSCs. Adipocytes are apparent in a variety of tissues, distinguished by a peripheral flat nucleus with a large white or brown fat droplet and low

eccentric cytoplasm. The number of these cells can be altered during the early years of life, and in adolescence, the volume of stored fat will change by fasting or overfeeding. Androgen and estrogen (sex hormones), in addition to resistin, leptin, and adiponectin, are synthesized by fat cells. These hormones influence the development of secondary sex characteristics, and leptin, along with two other proteins, influences energy balance and homeostasis with some appetite-suppressing effects. Recently, adipose has been shown to be a promising source of MSCs, and some researchers have isolated multipotent cells (9).

2.3 Biological characteristics of circulating MSCs

To date, researchers have not consistently agreed upon the criteria for the classification of MSCs. However, there are following criteria which have been suggested by ISCT, International Society for Cell & Gene Therapy, for categorizing precursor cells like mesenchymal stem cells antigen expression, adherence to certain culture plastic surfaces, and multipotent differentiation potential (12). These conditions are met by circulating MSCs. In 1997, Fernandez et al. isolated cells with the same specification of mesenchymal stem cells in some growth factor-mobilized PBSC (peripheral blood stem cells) derived from patients with breast cancer (17). These mesenchymal stem cells expressed fibronectin, collagen type 1 and type 3, CD73 (SH2 and SH3), CD54, and CD106, without expressing antigens CD34, CD45, or CD14 (Figure 2). In 2000, Zvaifler et al., have found the earliest precursors of the mesenchymal cell lineage, in the blood samples of a healthy population, which were known as PBMSCs, peripheral blood mesenchymal stem cells (18). These cells were also expressed BMPR IA, IB, collagen type 1, and vimentin but CD14-negative and CD34-negative, and CD45-negative (Figure 2) (18). They have detected fibroblast-like cells as well as small and round cells in these stromal samples, which were isolated from peripheral blood cells (18). Zvaifler et al. also demonstrated that the morphology of these progenitor cells is not dependent on culture conditions (18).

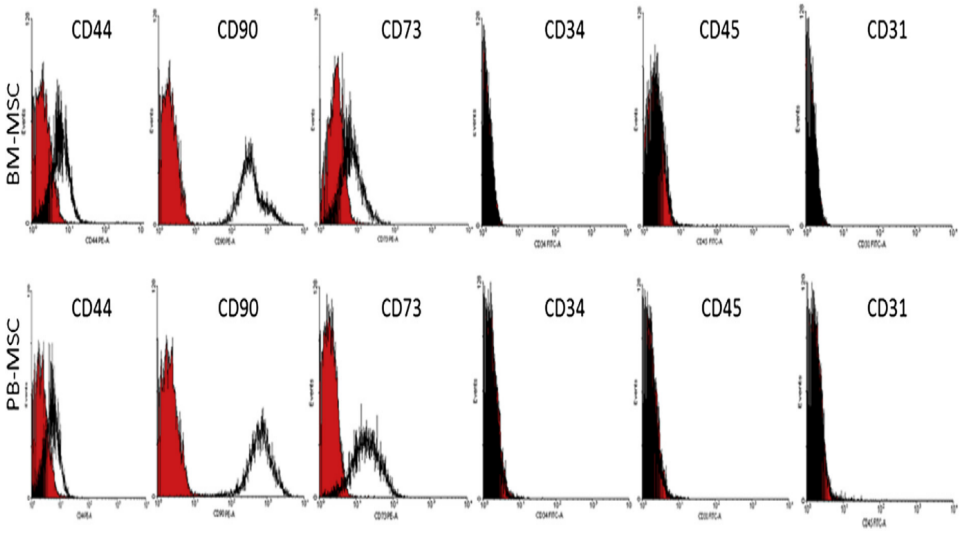

FIGURE 2. Adapted from Xu and Li (2014.), surface markers present on the BMMSCs (bone marrow mesenchymal stem cells) and PBMSCs (peripheral blood mesenchymal stem cells) were analyzed by FCM (flow cytometry). The red indicates Isotype controls, and the black indicates surface marker analysis done with antibodies (12).

2.4 Sources of MSCs

While bone marrow (BM) has the richest supply of MSCs, in actuality, MSCs comprise just a tiny fraction of the total cell count in the BM. MSCs, are plastic-adherent, and while easy to isolate from the BM, only make up one of every 10,000–100,000 nucleated BM cells (19). Even MSCs cells, which originated from individual donor's bone marrow, have different quality or yield (20).

MSCs are also found outside the bone marrow in other tissues of the human body, such as adipose tissue (21), amniotic fluid (22), fetal liver (23), umbilical cord blood, peripheral blood (18), and lung (24). As humans age, the numbers of MSCs decrease. Highest levels are found in neonates and infants, and this amount decreases during the lifespan to approximately fifty percent the count of neonates by the

age of eighty years (25). The most considerable number of MSCs are found during the first three months of life, falling to approximately $1x10^{-4}$ percent in the second three months and comprises just as much as $3x10^{-5}$ percent of nucleated cells found in the blood in the umbilical cord (23).

2.5 Isolation and marker specificity characteristics of MSCs

As we mentioned before, a significant source of MSCs is in the bone marrow. However, other cells which have similar properties to MSCs have been obtained from different tissues, including neonatal and fetal, as well as adult tissues, such as adipose tissue, compact bone tissue, peripheral blood, dental pulp, dermis, fetal lung or liver, umbilical cord blood, adult brain, amniotic fluid, skeletal muscle tissue, synovial membrane, circulatory system cells, and human islet cells (23, 24, and 26-31). The perivascular location for these MSC-like cells suggests MSCs function as pericytes, important vascular cells that wrap around the endothelial cells of microvasculature and capillaries present in various tissue and organs. Pericytes are mural cells around the blood vessels; the function is in the stabilization of blood vessels, regulate tissue homeostasis, and respond to damages imposed to the tissue by releasing immunomodulatory bioactive trophic compounds properties. Adventitial cells and pericytes have similar behavior to MSCs: their gene expression profiles are similar, they express mesenchymal markers, and they possess the same potential for development and differentiation (32). The current ISCT, International Society for Cell & Gene Therapy criteria, apply specifically to the hMSCs derived from bone marrow and may not pertain to those derived from another type of tissue. This is because the negative markers that used to classify them are expressed in the hematopoietic cells, which encompasses a significant portion of the bone marrow and are the main contaminating cells at the stromal culture derived from bone marrow (32). It has been reported that MSCs express CD29, CD44, CD71, CD73 (SH3 and SH4), CD90, CD105 (SH2), CD106, CD146, CD 166, STRO-1, and GD.

2.6 Differentiation potential of MSCs

It is possible to induce cultured MSCs in order to give rise to different tissues of mesodermal descent such as osteoblasts, adipocytes, and chondrocytes (Figure 3); however, the tissue's origin may affect the differentiation potential. The niche of stem cells can perform as a factor to stabilize the linage multipotential and self-renewal of stem cells. Isolation of MSCs from a specific tissue could result in a different subset of cells with diverse differentiation potentials. For example, MSC-like cells derived from the synovium have better chondrogenic potential in comparison to BMMSCs. This data is vital in terms of developing a therapeutic method in order to stimulate progenitor cells residing in the tissue for articular cartilage repair. A different investigation has compared bone marrow mesenchymal stem cells to mesenchymal stem cells derived from the pulp in the dental tissue. The study found that dental pulp-derived MSCs had superior differentiation capabilities to osteogenic cell lineages in comparison to BMMSCs, however, had shown fewer potentials in differentiating into adipogenic cell lineages. The inherent potency of mesenchymal stem cells to differentiate into different germ cells inside the culture should give pause. Evidence has shown that surface markers present on neural cells and smooth muscle cells have been expressed spontaneously by undifferentiated mesenchymal cells in the culture. The impact of characteristic tissue-dependent origins of mesenchymal stem cells on the abilities they have in differentiation to specific lineages needs to be considered when used for therapeutic applications (32, and 36).

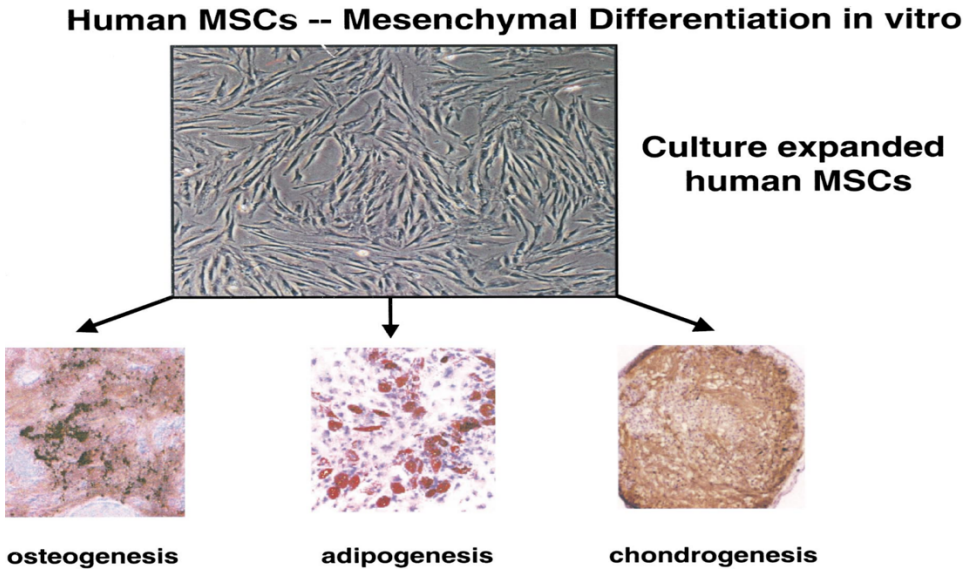

FIGURE 3. Adapted from Deans and Moseley (2000). Human MSCs differentiation in vitro. (36).

2.7 Basic biology and functions of hMSCs

The hMSCs form an inharmonious cellular colony that functionalities, as well as properties, rely on surrounding tissue specifications. They have the potential to expand to colony-forming unit fibroblasts (CFU-F) in culture, which possesses a prolonged ability to proliferate in vitro. In vivo, examinations on the rodents which have used [H]-thymidine as a labeling agent, proved that colony-forming unit fibroblasts are present in non-cycling conditions. The quantity of the harvested cell colonies by bone marrow aspiration depends on culture conditions and varies among species. For example, whereas adult human BMMSCs can form colonies, rodents BMMSCs require a source of irradiated feed cells (20). While the focus of numerous investigations, the characteristics of MSCs cultures need to be studied further. Previous studies suggested that mesenchymal stem cells, which were obtained from the bone marrow, contained phenotypically identical cells, forming spindle-like shape, symmetrical

colonies (up to ninety-eight percent homologous). However, latest investigations have demonstrated that colonies which are made up of a single type of cells are actually heterogeneous from the morphologic point of view, and they are comprised of at least two different cell types: large, cuboidal or flattened cells, which renew slowly, and small, spindle-shaped cells, which renew rapidly. These subtypes are thought to give all the mesenchymal stem cell populations the ability to develop and increase in the culture. Also, it was assumed that mature stem cells secrete substances that can stimulate the cycle of rapidly self-renewing. Accordingly, it was suggested that these cells represented a subgroup of recycling ex vivo as uncommitted. Still, recent studies have shown that colonies of mesenchymal stem cells consist of three different sorts of cells. The third group is thought that be comprised of small cells with fast self-renewal potency, and they may be the earliest progenitors with the most significant potential to multilineage differentiation. Their diameter is approximately seven micrometers and has an almost high nucleus-cytoplasm ratio. Proteins and specific surface epitopes, such as VEGFR2, RTKs, TfR, and annexin A2, are used to discriminate between these and more mature cells. Some of these cells that renew themselves rapidly express different markers too, such as a multidrug-resistance epitope, CD117 (c-kit), and EMA. Impressively, these cells show negative for STRO-1, which is thought to be a typical marker of MSCs (34).

2.8 Growth and development of MSCs

Many protocols have been introduced for the growing and expansion of mesenchymal stem cells. Those cells that primarily attach to plastics used for tissue culture, develop into symmetrical colonies of fibroblasts after five to seven days. Human MSCs expand quicker and maintain their maximal multipotency when they are cultured at lower concentrations. The seeding density of the cells is about $1x10^4$ - $4×10^5$ cell/cm^2. The density of the primary culture will affect both MSCs morphology and growth.

They will take spindle-like shape to themselves if cultured and grown in low densities, however after reaching higher densities, they forming several layers, and they will turn flat.

Mesenchymal stem cells can maintain their differentiating capabilities for twenty to thirty population doublings level. The immense potentials for proliferation in these cells are indicated by their ability to grow, divide, and population doublings over fifty times. Of course, their proliferative potential can be affected by a number of growth factors and cytokines (34, and 35). Inspection of the cell cycle profile of these cells has shown that almost ten percent of these MSCs exist in the phase M, G2, and S of the cell cycle, whereas the large percentage of them stay in phase G0 or G1. MSCs retain regular telomerase activity and a karyotype even in passage twelve. Even so, an extensive subculture of the MSCs disturbs their performance; eventually, they will show some signs of aging.

2.9 Potential of MSCs in self-renewal

A defining characteristic of MSCs is their ability to perpetually self-renew. Throughout an organism's lifetime, each cell will regenerate into identical copies of themselves, this will occur for some just for a portion of the lifespan, but for some, such as the progenitors of the immune system, for the entire lifespan. The potential of MSCs to self-renew remains an area of intense interest, and numerous approaches have been employed to discover to define the limits of their abilities. During in vitro serial multiplication, the BMMSCs has highly variable self-renewal capability and significant ability. Specific growth factors present in the serum are needed for the cells to be able to enter into the cell cycle and continue its development to form colonies. Higher proliferation (that is more than 50 doublings) was observed after adding some particular growth factors (for example, fibroblast growth factor 2) to the underlying cell culture media. The expansion potentials of MSCs is also dependent upon the initial cell seeding concentration (10, 11).

2.10 Multilineage differentiation potential of MSCs

Since it was first found that MSCs had the ability to trans-differentiate into many lineages, MSCs from a diversity of species have been studied. Both in vivo and in vitro, hBMMSCs have been shown to have the ability to form mesenchymal derived tissue such as adipose, bone, tendon, muscle, cartilage, and hematopoietic-supporting stroma (Figure 4). This ability has made them the premier candidate in order to study and learning strategies for regenerating tissue under clinical conditions, such as repairing local tissue, augmentation and regenerating tendons, cartilages, and bones (11).

The ability of even a single colony derived from a single predecessor to differentiate into multilineages suggests that MSCs are heterogeneous. For example, only one-third of the primary adherent BMMSCs population is pluripotent (i.e., chondrogenic, osteogenic, and adipogenic). Other investigations using purer colonies of MSCs induced in vitro and conditionally immortalized clones have also shown this heterogeneity, supporting this theory, the colonies of the cells which derived from mesenchymal stem cell are heterogeneous, with consideration their development-potential (11).

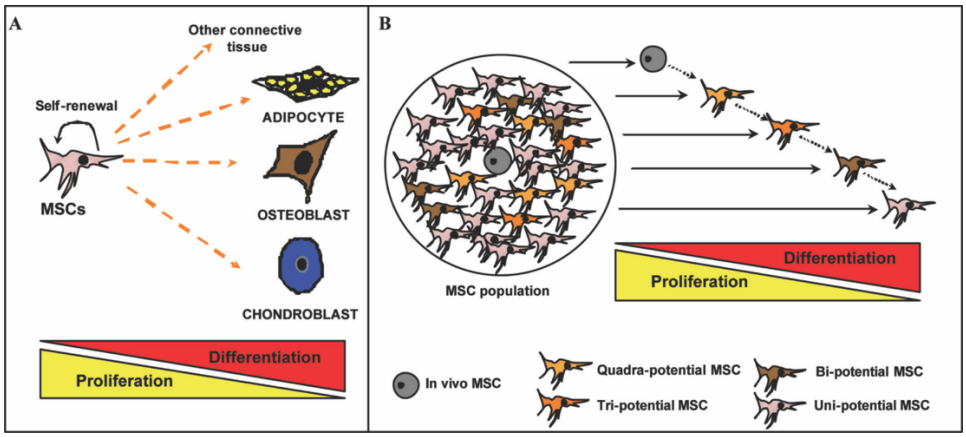

FIGURE 4. Adapted from Baksh et al. (2005). Models for differentiation of MSCs. (A) Differentiation of an MSCs into all types of connective tissue cells (B) An alternative demonstrating subpopulations in MSCs with varying potential for differentiation (11).

In vitro and in vivo data supports the presence of subpopulations of MSCs at different states of differentiation within the bone marrow. As depicted in Figure 4, different types of multipotent mesenchymal stem cells have a self-renewing capacity and will lead to particular types of cells with resultant differentiated phenotypes. During long-term culture, multipotent MSCs, which are present in low frequency, are depleted more rapidly than the more mature and higher number MSCs. It is still unclear how these MSCs with high multipotential capability are remained in vitro culturing samples (10, and 11).

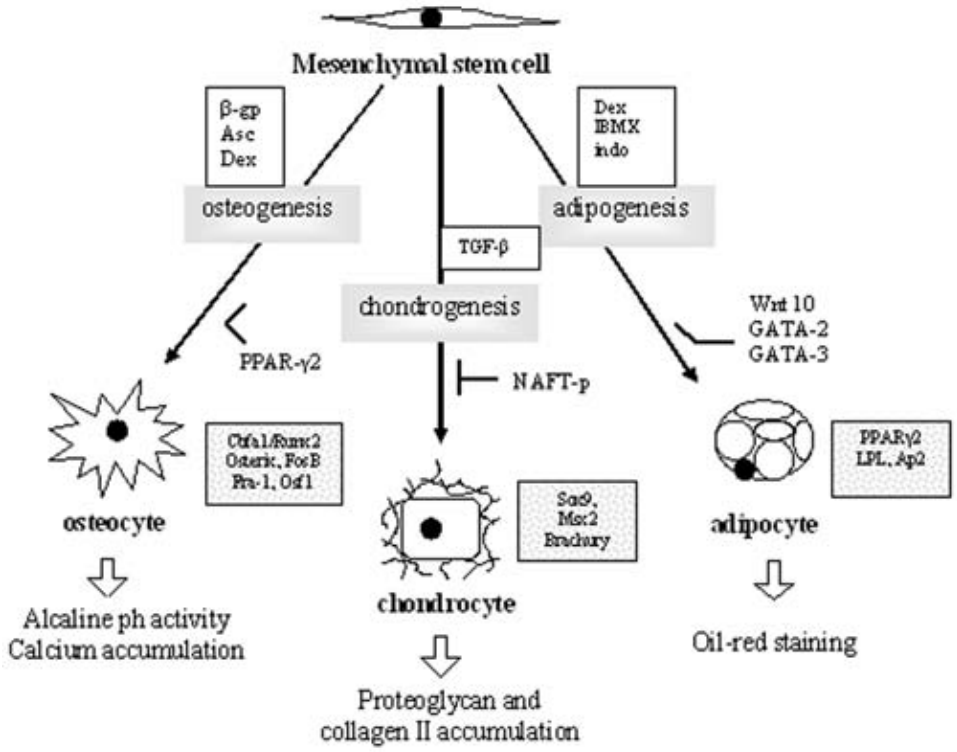

FIGURE 5. Adapted from Bobis et al. (2004), A schematic for MSC differentiation into three lineages. The squares at the top list demonstrating the factors needed for induction each pathway; the squares (shadowed) at the bottom list the main factors for transcription. Empty arrows denote possibilities to recognize the cells after differentiation. Abbreviations: aP2 for fatty acid-binding protein), Asc for ascorbic acid, β-gp for β-glycerophosphate, Dex for dexamethasone, IBMX for isobutylmethylxanthine, indo for indomethacin, LPL for lipoprotein lipase, NAFT-p for the nuclear factor of activated T cell, PPARγ2 for peroxisome proliferation-activated receptor γ2, TGF-β for transforming growth factor-β (37).

2.11 MSCs and Regulation of differentiation

Another feature of MSCs that makes them a promising candidate for many therapeutic applications is their ability to take up the specifications of the cells that will be driven out of the embryonic mesoderm, ectoderm, and neuronal cells under defined inducing conditions (Figure 5). While the precise mechanisms which rule MSCs differentiation have not been completely elucidated, various studies have provided some genomic and genetic information to form a model of regulation. This model is suggestive of two continuous, yet distinct, compartments. MSCs undergo transcriptional modification in the first compartment, which helps them to generate precursor cells that show no changes in their phenotype and their self-renewing capacities. Most of the MSCs which have been cultured in vitro will remain still similar to the ones that are present in the bone marrow of adult individuals until stimulated, for example, with growth factors. Once stimulated, the multipotent MSCs undergo asymmetric division, producing two daughter cells, one which retains the multipotency and the other becomes a progenitor cell that has a more restricted development. The blast cell proceeds to its symmetrical division, producing more blast cells that are tri-potent and bi-potent. The morphology of these cells is very close to the mesenchymal stem cells with multipotential capacity, but they have a different transcriptional program, and therefore will consist of the stem cell niche. As these precursor cells start to divide symmetrically, they produce uni-potent progenitor cells, which eventual transition from the stem cell niche to the engagement niche, and finally, they become terminally differentiated (11).

The traditional view of the sequential and orders of the stem cell progression from one phase of differentiation to the further phases while determining their phenotype was called into question by current studies, they have found that adult stem cells are able to produce cell different to their tissues of

origin during transplantation in vivo. These studies showed that extracellular stimulating signals could reprogram chondrocytes, adipocytes, and osteoblast with complete differentiation, which originated from MSCs. Through this procedure, cells that were once committed to one cell type undergo extensive proliferation, and then de-differentiate to a primitive stem cell-like state. Then these cells can be induced to differentiate into a completely new phenotype. This process, known as "phenotypic switching," is suggestive of the retention of multipotentiality by precursor cells and also by cells with full differentiation. In vitro analyses have been able to identify important factors that drive MSCs into particular lineages and remain the commitment to the differentiated phenotype. These include intracellular transcription factors (for example, PPARγ, MEF2, CBFA1/RUNX2, and Sox9), extracellular matrix molecules (for example proteoglycans, and collagens), molecules that have been secreted, and the receptors they bind to (for example TGF-β), and actin cytoskeleton (11). One area of great interest is in the replacement of cartilage produced by stromal cell precursors. BMMSCs precursors have been induced in vitro to differentiate into cells with chondrogenic potential, and implantation of cells derived from MSCs has been successful in repairing damaged cartilage. Recent reports of differentiating MSCs into tendons have also been published. These findings are exciting because it had been thought that MSCs lineage boundaries were defined and inflexible, but these results suggest greater plasticity. Further investigation will be needed to understand better the potential and limits of the connective tissue stem cell hierarchy (32).

We do not know it clearly yet whether there is a single MSC with multipotential ability that results in each mesenchymal cells or whether there some combination of progenitor cells committed to different cell lineages. To date, this question has not been answered by in vitro and animal implant studies: the studies show different, often opposing, results. Previously, it was thought that MSCs were limited to differentiation only into tissues of mesodermal origin. Recently, this belief has changed on the basis of

findings from large-scale MSCs biology studies. Successful differentiation in a diverse lineage of cells has been achieved, inclusive adipocytes, fibroblasts, osteoblasts, chondrocytes, cardiomyocytes and myoblasts, cenocytes, tenocytes, neurons, and hepatocytes. Some scientists, however, hypothesize that the generation of cells with other origins than mesoderm is because of particular gene expression with processes that cause reprogramming in MSCs or is due to specific soluble factor activity (20, and 34).

2.12 Clinical application of MSCs

This extensive proliferative ability and potential of MSCs to differentiate into different types of cells such as fat, cartilage, and bone, making them an interesting model in regenerative medicine. The Number of clinical experiments focusing on the applying of mesenchymal stem cells has been raised, and it has been made an improvement and advances in fields such as cell biology and gene therapy. In clinical practice, MSCs may be isolated from different tissues, expanded in large numbers in culture, genetically engineered, and reintroduced into a patient. To ensure full healing, the regenerated tissue needs to be integrated into the host tissue, which will surround it and differentiated over natural pathways of signaling. If infused systematically, MSCs can engrave into different organs, and different tissues, this type of grafting has proved long term success. Peripheral circulation infused MSCs in animal experiments with myocardial infarction, cerebral ischemia, and fracture of the bones have demonstrated that they can migrate to the site of injury. Eighteen days after the infusion of MSCs into mice babies, the scanning of the whole body using special imaging system demonstrated that the most of the infused cells had been found in the liver and the lungs of the mice, and only a tiny proportion was found in other parts. Thirty-five days later, a noticeable amount of cells were found in the bones, suggesting that they may be involved in the formation of the bones. Engraftment seemed to happen at quite a low level and vary across different tissues. This investigation also demonstrated the presence of

two populations of MSCs: the small-sized, rapidly self-renewing, and the larger, slower renewing MSCs. The two subpopulations also expressed unique surface epitopes with the more rapidly self-renewing MSCs expressing CXCR1 (C-X-C motif chemokine receptor 1) as well as CXCR4 (C-X-C motif chemokine receptor 4), which accepted to participate in the trafficking of MSC (34, and 35).

Many sources have proposed mesenchymal stem cells as a great potential tool for gene therapy. These can undergo profound genetic changes, for instance, that can be transduced by viral vectors which are carrying a therapeutic sequence of gene or cDNA. Mouse studies using genetically modified MSCs have shown an approximately seventy-four percent stable gene transfer efficiency after primary implantation into an ectopic site and a subsequent transplant into a second donor. Therefore, MSCs could be used to target specific genes into organs or tissues of specific needs. Ongoing clinical studies are investigating the use of mesenchymal stem cells, which are implanted with viral vectors that contain the genes coding for coagulation factor VII or factor IX to treat hemophilia. Treatment of bone defects is one of the potential applications of MSCs in regenerative medicine. New bone repair strategies induce bone formation by recruiting local MSCs to biodegradable scaffolds impregnated with recombinant BMPs (bone morphogenetic proteins). This method was successful in an examination of animals and showed attraction of the MSCs toward bone morphogenetic protein; two were able to cause regeneration in damaged bone. Nevertheless, the findings were not completely satisfactory as the quantity and quality of regenerated bone remained inconsistent. MSCs were triggered in another instance by human bone morphogenetic protein two genes, which were mediated by adenovirus and injected into nude mice intramuscularly. This also led to MSC proliferation and differentiation. These studies suggest that improvement in outcomes may be achieved through the aid of other common cytokines such as IGF, PDGF, and FGF (34).

The use of MSCs in Osteogenesis imperfecta (OI) treatment should be given special attention. Osteogenesis imperfecta is a kind of genetic disease that results from mutations in a gene coding for collagen type 1 and causes various abnormalities, particularly in bone structure. More than 150 mutations in COL1A1 and COL1A2 have been shown to be associated with OI. Collagen is the bone's main extracellular matrix protein, and OI patients suffer from frequent and multiple fractures, progressive limb and spine deformities, impaired bone growth, and short stature. Thus, most OI treatment methods aim to improve bone strength by enhancing collagen's structural integrity. For all OI therapies, the only beneficial outcomes are produced by cell and gene regimens, and these methods tend to be the only appropriate tools. This approach to cell therapy aimed at the development of osteoblasts from mesenchymal stem cells was initially studied in murine models. The MSCs samples were taken from the transgenic mice and then grafted into recipient mice, which was irradiated. These cells were monitored, and their location was observed for one to five months after infusion. Between one-and-a-half and twelve percent of the tissues, including bones, showed the presence of the transplanted cells. Other studies using an immunodeficient SCID (severe combined immunodeficiency disease) mouse model also demonstrated that hMSCs had the ability to home and differentiate into osteoblasts in vivo (34).

2.13 MSCs and tissue repair

The Discovery of mesenchymal stem cells has exciting clinical implications due to their multipotency, role in immunosuppression, and immune privilege. Furthermore, the use of MSCs does not raise the same ethical issues as ESCs. Adequate findings exist to support the use of MSCs in the treatment of a wide area of disorders, and indeed plenty of reports exist about the current utilization of MSCs for the regeneration of cartilage and bone tissue (12). Local delivery of BMMSCs in combination with a scaffold made from microporous-hydroxyapatite has resulted in significant advances due to the repair of large

long bones defects (12). Another such example exists, caprine-model of OA (osteoarthritis); local treatment of injured joints with MSCs led inner meniscus to regeneration: the marked MSCs were observed in the regenerated tissue, and the usual further degeneration was reduced (36). Another promising area of research that has shown success in the management of osteochondral lesion and defects in rabbits is the loading of bone marrow, whereby an artificial scaffold is mixed with bone marrow. It seems that the loading of bone marrow accelerates primary stages in the process of repairment (12).

Evaluation of fracture healing in a mouse model showed that systemically applied MSCs extensively enhances cartilage-bone content and fracture repair (37). The intravenous application of MSCs has been used to treat aseptic necrosis of the hip in rabbit models; the MSCs migrate directionally to the site of necrosis and survive without any sign of immunological rejection (38). In large part, the MSCs were obtained from bone-marrow (BMMSCs) or other tissues, while PBMSCs (peripheral blood-derived Mesenchymal stem cells) were rarely utilized (12).

2.14 MSCs capacity in tissue regeneration and wound repair

MSCs will home to wound sites when administered exogenously — to primarily inflamed or damaged blood vessels (Figure 6). MSCs produce a complex set of soluble mediators, almost "sensing" environmental requirements in a medium-specific manner. Such products facilitate angiogenesis, regeneration, remodeling, activation or suppression of immune cells, and cell recruitment. The MSCs are located in the perivascular region (pericytes) and do not demonstrate the same responses in all tissues, because all tissue microenvironments are different: this can influence the efficacy and potency. Thus, the ability of MSCs to function is dependent upon the protocol for isolation and expansion by which the MSCs preparation is based and must take into account the injury-specific context in which

MSCs are placed. The ability of the MSCs to respond to a given environment or an altered environment is achieved through various control mechanisms that include transcriptional regulation and signal transduction mechanisms involved in tissue repair and immune response. The process of repair involves regulating the deposition of the extracellular matrix, collagen synthesis, proliferation of fibroblasts, and activation of platelets, fibrinolysis, and angiogenesis. The immune response also includes suppression of T-cells, recruitment of neutrophils, and activation of macrophages. Investigations also indicated that MSCs' ability in regulating immunity is characterized by MSCs produced soluble products that promote changes in the status of immune cell activation. MSCS products, like the ones working as the immunity mediator, may include antimicrobial proteins, inflammatory mediators, and cytokines (39).

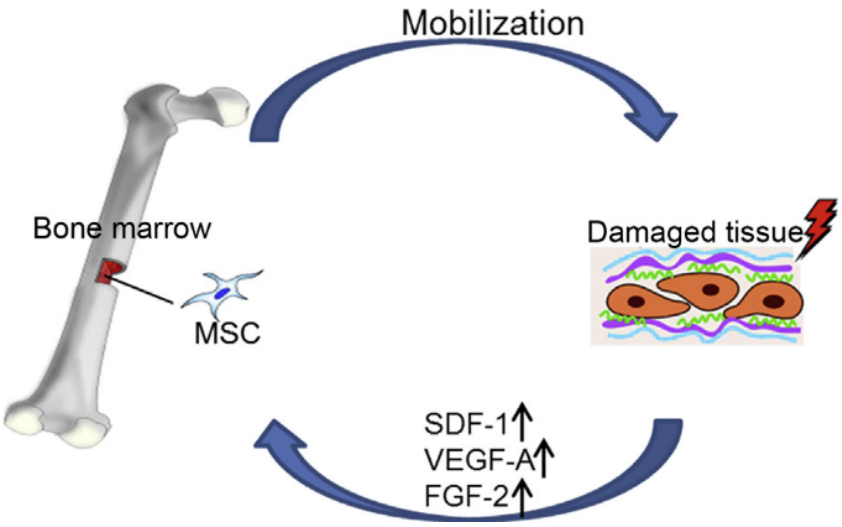

FIGURE 6. Adapted from Xu and Li (2014). Illustration demonstrating the mobilization and migration of mesenchymal stem cells to the injury site (12).

The in vivo mechanisms of repairing of the damaged tissues by MSCs are not well unknown. The latest studies are indicating that paracrine factors that are secreted by MSCs are involved in the repair process, instead of transdifferentiating MSCs into specific types of tissue cells. To support tissue repair, neovascularization and an increase in angiogenesis may result in paracrine secretion by MSCs. For example, in myocardial ischemia of the mouse and pig samples, minimization of infarction volume has been achieved by supplementation with exosomes absolve samples taken from cultured tissue, which were conditioned by MSCs driven from a human embryonic stem cell. Transplantation efficacies using mesenchymal cells from different tissues and conditions of donor and MSCs culture have also been investigated. These animal models of lung injury, myocardial infarction and kidney injury illustrate the heterogeneity of MSC subpopulations and may support the use of MSCs in tissue damage and neurological diseases. Heterotopic ossicle transplants can be used to assay the cells' intrinsic capacity to generate different tissues in animals and represent the functional ability of MSCs in vivo. To address the issue of MSCs self-renewal, the feasibility and reproducibility of serial transplantations of mesenchymal stem cells taken from an initial ossicle need to be assessed. Animal models and rigorous in vitro models are essential to understanding the function and nature of the MSCs, and the mechanisms responsible for tissue repair mediated by MSCs. Knowing the function and biology of various MSCs subgroups working in the process of tissue repair is crucial in evaluating the potential applications they may have for specific therapeutic purposes (32).

2.14.1 Bone and cartilage repair

Devine et al. first demonstrated that non-human primate bone marrow could be contained in cultivated MSCs (40). Using a rat model of bone repair, MSCs were kept in a cylinder with pores and then implanted in the femur of the rat, then it was determined that after eight weeks, there was complete healing in the defect containing the implant. This result demonstrated that MSCs expanded in the

culture could retrain and take part in the formation of new bone in vivo. Nonetheless, osteogenic sites cannot be home to culture-expanded MSCs. Two approaches to address this problem have been explored. First: peptidomimetic ligands for integrin beta-1 on the surface of mesenchymal stem cell have coupled with bisphosphonate in order to promote migration to the bone surface and second: RNA interference targeting casein kinase 2-interacting protein-1 (CKIP-1), negatively affects osteogenesis by using (AspSerSer)(6)-liposome to target Runt-related transcription factor 2 for bone degradation. The recent approach was the primary method able to promote bone resorption, lacking associated activation of the osteoclasts and resorption of the bones. Culture-expanded allogeneic MSCs were also used to treat children with OI in clinical trials. It has shown recovery in these patients as demonstrated by decreased bone fracture incidences during the first year after MSCs engraftment; Although over time, the effects were reduced. This reduction could be due to the possible culturally expanded cells aging or terminal differentiation while culturing the cells and passage, which may have been associated with epigenetic changes in MSCs during prolonged culture (32).

Cellular allographs containing MSCs were used for bone repair purposes in the high-risk foot and ankle surgeries. The allografts containing MSCs showed increased healing and decreased time for fractional weight-bearing conditions. In rabbit cartilage defect models, MSCs are also documented to promote cartilage repair using nanofiber-based electrospun technology. A study of transplantation that used MSCs with fluorescent-labeled showed that mesenchymal stem cells could locate to bone injury areas independent of their route of administration (32).

2.14.2 Muscle repair

Recently, a population of cells negative for CD90, and positive for CD73 and CD105 was isolated from muscle tissue of humans; they were used as markers to identify multipotent human muscle resident

MSCs (hmrMSCs). The previous investigation of heterotopic ossification (HO), a skeletal-muscle disease, has shown that nonmyogenic precursor cells playing a role in the formation, regeneration, and development of irregular tissue. The samples were taken from skeletal-muscle of the patients with heterotopic ossification and the control group without any skeletal-muscle disease, the samples have been exposed to collagenase, and then the resultant cells have been moved into culture medium utterly free from an animal- and human-derived components (xeno-free) before they have been chosen in colonies depending on the expression of CD90. The cells' potential for adipogenic, osteogenic, and chondrogenic differentiation was furthermore assessed. The population of hmrMSC negative for CD90 demonstrated strong differentiation of chondrocytes and osteogenic cells, adipocytes (mitochondrial brown fat uncoupling protein-1). The study demonstrates that in human heterotopic ossification, there are adipocytes positive for UCP1 and confirms that adult skeletal muscle hmrMSCs may be precursors to all cell lines in heterotopic ossification (32).

Observations also suggest the utility of MSCs transplantation after myocardial infarction in myocardium regeneration. Among all of the cell populations derived from bone marrow, only mesenchymal stem cells have demonstrated the ability to differentiate in cardiomyocytes. 5-aza-cytidine has been used to induce the differentiation of cardiomyocytes, verifying that this type of cell may be derived from MSCs. Not only did the cells contain structures similar to myotube and myofilaments, in the investigations carried on by staining, they were also showed traces of specific markers for cardiomyocyte such as desmin, actinin, sarcomeric myosin, and expressed specific genes and transcription factors for cardiomyocyte. A similar effect was observed in the case of hMSCs: taking inspiration from in vitro studies, some in vivo experiments were performed by researchers, and when hMSCs were injected into SCID mice hearts, a small percentage (0.44 percent) of the cells engrafted. MSCs successfully colonized the damaged tissue when used in animal cardiac injury models and converted it into properly

functioning cardiac cells. Similarly, when MSCs transduced with an Akt-encoding virus were transplanted into an injured heart, the left ventricle's pathological remodeling after infarction was prevented. About eighty percent of the injured myocardium has been regenerated and fully regained its function. MSCs have been able to increase the weight of the ventricular wall in addition to increasing cardiac function. Also, administrating the MSCs locally into the heart tissue produced newly formed myocardial, raising the hope for the application of these cells in the treatment of coronary heart disease. The injection of MSCs into the area of infarction in patients after myocardial infarction seemed to improve the overall functioning of the heart (34).

3 Arthroscopy

Arthroscopy is a minimally invasive surgical technique used in orthopedic surgery for visualizing, diagnosing, and treating joint problems. Two Greek words originate from the word "arthroscopy": "arthro" (joint) and "skopein" (to look), meaning to look inside the joint. Arthroscopic surgery is a widely applied procedure amongst orthopedic surgeons.

Arthroscopic surgery begins with a small incision, and small tools, including a lens and lighting systems, are utilized to visualize structures in the joint. Fiber optics move light into the joint at the end of the arthroscope. The arthroscope is connected to a miniature TV camera, and a tiny incision allows the surgeon to see the inside of the joint. The camera is set on the arthroscope, and it will show the joint space on a monitor, it makes the specialist able to see the joint's cartilage, ligaments, and other components. The surgeon then determines the severity or sort of damage and injury, if necessary, to fix and rectifies the issue (41). Therefore, it is crucial to understand the essential principles of arthroscopy. Arthroscopic procedures, in some cases, have better outcomes because the smaller incisions result in

less structural damage, reduced pain in the immediate postoperative period, and faster recovery for patients compared to open surgical techniques (42).

3.1 How to perform arthroscopy

While arthroscopic surgery is less severe than traditional open surgery, anesthesia and advanced tools are still required in an outpatient surgical unit or operating room in the hospitals. Based on the joint or injury to be treated, one of the local, spinal, or general anesthetic methods is needed.

A small incision is made, into which the arthroscope is inserted (about the width of a buttonhole). If other areas of the joint must be visualized, other tools or several other incisions may be made. If needed, surgery is done by using instruments that are specially designed, and they are implanted through additional incisions into the joint. In the beginning, arthroscopy was performed with diagnostic purposes before standard open surgical planning. However, the advent of advances in instrumentation and surgical techniques have made arthroscopy the treatment of choice for many conditions, such as meniscal tears.

The small incisions are covered with a dressing after arthroscopic surgery, and patients are moved from surgery to a recovery room. Most patients at this time require little or no medicine for pain. Upon discharge, patients are given instructions on how to take care of the incisions, what tasks should be avoided, and what exercises will help recovery. The surgeon should inspect the incisions during the follow-up appointment, remove any sutures present, and evaluate the rehabilitation program. The type of surgery needed, and the length of recovery time depends on the problem's severity. Occasionally, during arthroscopy, the surgeon may find that the injury or disease cannot be adequately treated with arthroscopy alone; more extensive, open surgery may be performed while the patient is still

anesthetized or maybe implement at a later date after the patient discusses the findings with the surgeon (41, and 42).

3.1.1 Bleeding control during arthroscopy

It can be challenging to control intraoperative bleeding during arthroscopic procedures in the particular knee and shoulder arthroscopy. With varying degrees of success, direct (e.g., thermal electrocautery) and indirect (e.g., hypotensive anesthesia, arthroscopic pump) means of bleeding control were used (43), and several methods are used during arthroscopy to manage bleeding. A tourniquet, which is successful but also associated with complications, is one form widely used by orthopedic surgeons (44, 45, and 46). An investigation by Burkhart et al. showed that turbulence management by controlling bleeding during arthroscopy could improve visualization (44). Another method is to use an irrigation pump system or to increase the flow of the infusion fluid to avoid intraarticular bleeding which covers the surgical view; this method involves the use of intermittent suction on the cannula of the arthroscope or the insertion of a separate drainage portal (45, and 47). The application of local hemostatic agents to the irrigation fluid is a promising alternative method to prevent bleeding. During arthroscopy, intra-articular washing with a vasoconstrictor was shown to be effective in preventing intraoperative bleeding (48-51). Epinephrine is currently the most commonly used vasoconstrictor for this purpose (48-51).

3.1.2 Application of a vasoconstrictor for intraoperative bleeding control during arthroscopy

As mentioned, during arthroscopic procedures, a vasoconstrictor may be applied to the irrigation fluid to prevent intraoperative bleeding. Applying epinephrine into the fluid used for irrigation during arthroscopy of the shoulder joint showed significant positive changes in the visualization by surgeons (48). Another research by von Montfroort et al. found that in most of the common therapeutic arthroscopies performed on the shoulder joint, the use of epinephrine in a concentration of 0.33 mg/l

adding to fluid irrigation will noticeably improve the operative visual field (50). Significant reductions were also observed in the duration of the operation and consumption of the irrigation fluid. It has also been shown that norepinephrine is a promising alternative to intraoperative bleeding control of epinephrine (52). Until now, during arthroscopy, epinephrine has been the most common medication used as a vasoconstrictor (48-51). Epinephrine is mostly used at 0.33 mg/l concentrations (50 and 52). In our center, the Department of Orthopaedics, Physical Medicine and Rehabilitation, University Hospital, LMU Munich, we dilute an ampule of epinephrine, which contains 1 mg of epinephrine, in 3 l of 0.9% NaCl irrigation fluid. We use this irrigation mostly for shoulder arthroscopy.

3.2 Arthroscopy and Mesenchymal stem cells (MSCs)

As we explained before, stem cells play crucial roles in tissue repair. During some arthroscopic surgeries, this role would be more critical. Preforming the arthroscopy, especially with microfracture, ACL-Reconstruction, meniscal-rapier, tendon repair, etc., this role is very prominent. Through this kind of method, the stem cells are moving out from bone marrow und starting the tissue repair process, as mentioned before (32, 39).

4 Research hypothesis

As mentioned before, hMSCs have a vital role in the regeneration of damaged tissue, including cartilage, fibroblast und tendon tissue (11). We wanted to investigate the possible toxicities associated with hemostatic agents used intraoperatively by arthroscopy. Until now, the most common local hemostatic agent to control the intraoperative bleeding during arthroscopy has been epinephrine. However, no studies have examined if exposure to this hemostatic agent may be toxic to hissed lead to cell lysis. This condition, which eventually leads to hMSCs death, ultimately results in decreased tissue

regeneration and progressive joint degeneration. This is especially important after microfracturing, ACI (autologous chondrocyte implantation), OATS (Osteochondral Autograft Transfer System), meniscal, and tendon repairs or reconstruction surgery. Vasopressin may be useful as an alternative to epinephrine for controlling local bleeding (53), it has been evaluated as an effective vasoconstrictor (54-57) but not yet evaluated for improving the visual field during arthroscopy: previous investigations have only concentrated on how the epinephrine can affect the visual field during arthroscopy (48-51). Previous studies have also examined the cytotoxicity of local anesthetics on hMSCs (58-60), but none has yet evaluated the cytotoxicity of local hemostatic agents on these cells. Since hMSCs are crucial to tissue regeneration, we were interested in assessing any adverse effects of epinephrine and vasopressin on hMSCs. In this in vitro experiment, we aimed to assess any potential cytotoxic impacts of epinephrine and vasopressin on hMSCs. We hypothesize that, if epinephrine is toxic to hMSCs, vasopressin could be a suitable alternative. If this hypothesis is correct, epinephrine should not be used during arthroscopy, which may be performed for cartilage repair, chondrocyte graft implantation, meniscal repairing, or tendon reconstruction, etc..

5 Material and Methods

The materials and methods used here have already been used in previous studies conducted by our research group. We have already performed this method as established in our Laboratory for Biomechanics and Experimental Orthopaedics, Department of Orthopaedics, Physical Medicine and Rehabilitation, University Hospital, LMU Munich. The methods utilized in this study were based upon those methods used by Ficklscherer et al. (59).

5.1 Cell culture

We obtained hMSCs (Lonza, Walkersville, Maryland, USA) from 38 male donors. These cells were harvested and cultured from bone marrow. The hMSCs were delivered in passage three and were CD29-positive, CD44-positive, CD105-positive, CD166-positive and CD14-negative, CD34-negative, and CD45-negative; they were capable of differentiating into one of the chondrogenic, adipogenic, and osteogenic lineages. These hMSCs had a cell viability of eighty percent. We did not want to force the cells to differentiate into osteogenic, chondrogenic, and adipogenic lineages, because it is known that fibroblasts and chondrocytes are both crucial for joint regeneration. If the vasopressin or epinephrine is harmful to primary hMSCs lineage, all sub-phenotypes would be affected. Thus, we decided to work directly with primary hMSCs. We used triple-layer culture flasks (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) (Figure 7) to seed the cells in alpha modified Eagle's medium (Alpha-MEM; Alpha-Medium, Biochrom GmbH, Berlin, Germany). The seeding density of the cells was $1x10^4$ cells/cm^2.

The medium contained ten percent of FBS (fetal bovine serum) (Biochrom GmbH, Berlin, Germany), fifty units/ml of Pen-Strep (Biochrom GmbH, Berlin, Germany), and L-glutamine (Biochrom GmbH, Berlin, Germany). This is the standard medium we are using to the culture of hMSCs, which has been described in our Laboratory for Biomechanics and Experimental Orthopaedics, Department of Orthopaedics, Physical Medicine and Rehabilitation, University Hospital, LMU Munich and is the same medium that Ficklscherer et al. used for their experience (59).

We put the cells in an incubator at 37°C with 5%CO_2 for ten days. We used phase microscopy to visually check the cells, each time the medium was changed; in this way, we assured that the hMSCs had proper cell morphology consistent with stem cells, and we also excluded any contamination. We

have regularly changed the culture medium every two to three days, and the cells were returned to the incubator with 5%CO_2 at 37°C. At eighty percent confluence, we have passaged the hMSCs with the help of $5x10^{-2}$% trypsin that contained $2x10^{-3}$% EDTA (Biochrom GmbH, Berlin, Germany).

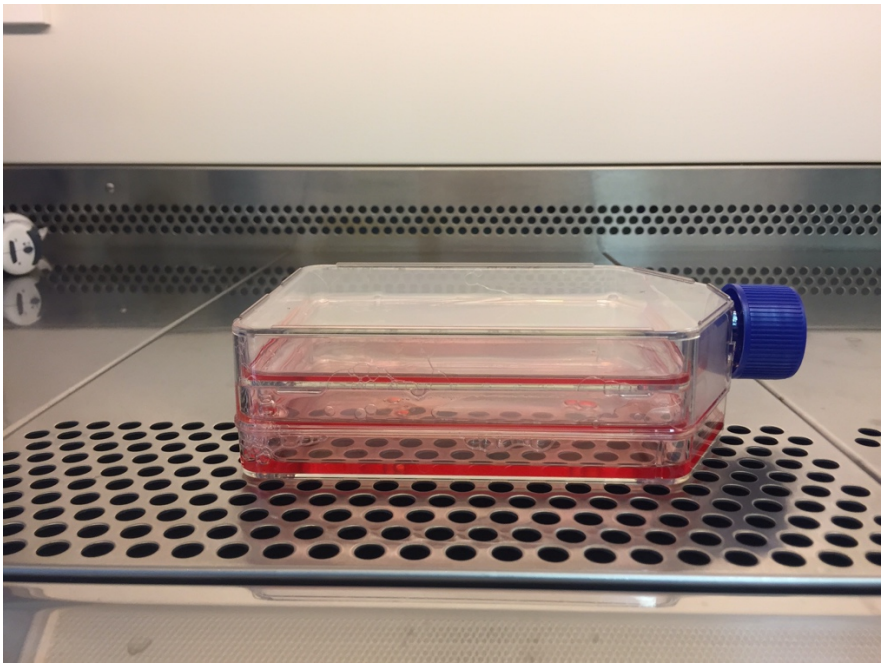

Figure 7. Triple-layer culture flasks, including hMSCs and culture medium.

5.2 Cell Count

To perform this step, we removed the media, washed the cells with PBS (Biochrom GmbH, Berlin, Germany), and then added $5x10^{-2}$% trypsin that contained $2x10^{-3}$% EDTA-Solution (Biochrom GmbH, Berlin, Germany) to the culture flasks. Then the cells were placed in the incubator at 37°C with 5%CO_2

for five minutes, and then fresh media was added to inactivate the trypsin. Cells were then spun in the centrifuge for five minutes.

After this step, we removed the medium and trypsin, and then the new medium was provided to the cells. Then Trypan Blue (TB) at a concentration of 1 to 1 (1 ml TB and 1 ml medium containing the cells) was added, and cell count has been performed with the help of a hemocytometer counting grid (Neubauer-chamber's) under the phase microscope. We performed the experiments at passage 5.

For the proliferation assays and mitochondrial activity (WST-1), we seeded the cells inside the 96-well cell culture plate (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) (Figure 8) at a concentration $5x10^3$ cells for each well (Table 1).

For RNA analysis, we seeded the cells using the 6-well cell culture plate (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) (Figure 9) at a concentration of $1x10^5$ cell for each well (Table 2); fresh medium was added to each well.

We evaluated a single 96-well cell culture plate and a single 6-well cell culture plate for each period (one hour, twenty-four hours, and seven days). Cells were returned to the incubator at 37°C with 5%CO_2 for one day.

5.3 Drug exposure

In the Department of Orthopaedics, Physical Medicine and Rehabilitation, University Hospital, LMU Munich, our standard irrigation fluid for arthroscopy of the knee or shoulder is 3 l 0.9% NaCl (B. Braun Melsungen AG, Melsungen, Germany). For controlling the local bleeding during shoulder arthroscopy,

we add one ampule of epinephrine, which contains 1 mg epinephrine to 3 l 0.9% NaCl to enhance hemostasis during the procedure.

This is the same concentration von Montfroort et al. (50) and Chierichini et al. (52) used for their studies. For this experiment, we used the same concentration for both epinephrine and vasopressin. We added one ampule (1 mg) of epinephrine (InfectoPharm drugs and Consilium GmbH, Heppenheim, Germany) and one ampule (20 units) of vasopressin (Pitressin, JHP Pharmaceuticals, Rochester, Michigan, USA), to two different containers, each containing 3 l isotonic 0.9% NaCl (B. Braun Melsungen AG, Melsungen-Germany).

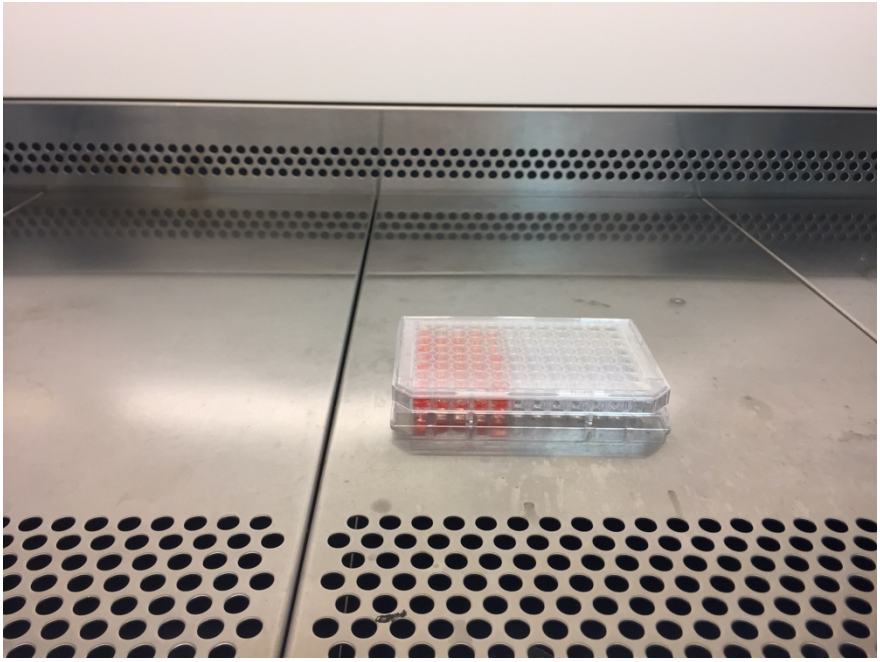

Figure 8. A 96-well cell culture plate.

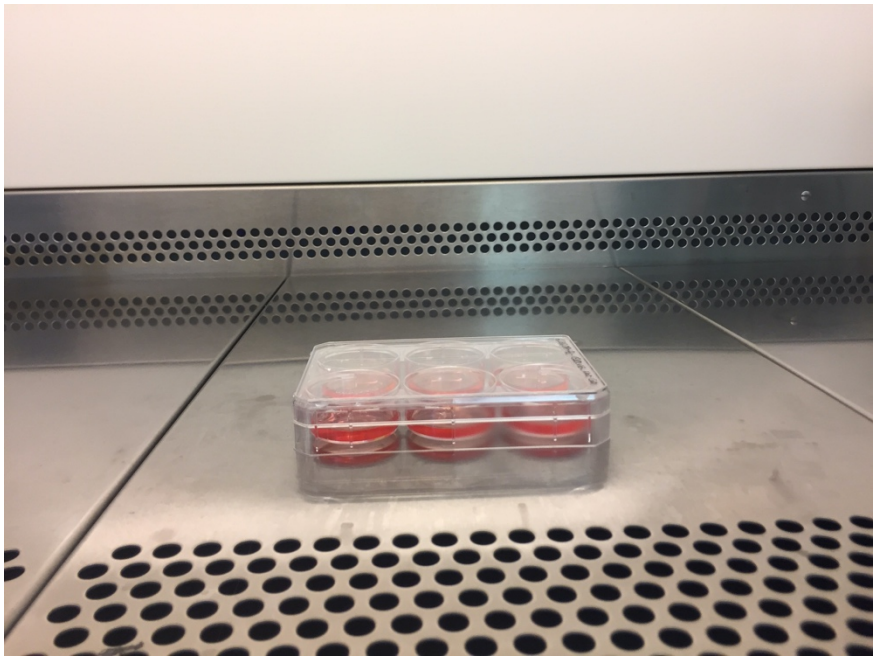

Figure 9. A 6-well cell culture plate.

We divided the cell cultures into subgroups that each consisted of 6 wells, with $5x10^3$ cells per well: the groups were exposed to either epinephrine or vasopressin 0.033% (1 ampule in 3 l 0.9% NaCl). We treated one subgroup with alpha-MEM-only, which was identified as the control group (Table 1). We obtained this protocol from Piper et al. and Ficklscherer et al. (59, 61, and 62). Briefly, the culture medium (alpha-MEM) was fully aspirated; 200 µl of vasopressin or epinephrine solution was added to each well in the respective subgroups. Next, alpha-MEM was added as a control. Cells have been placed back in the incubator with 5%CO_2 at 37 °C for forty minutes. After that, we removed the solution of epinephrine or vasopressin and added a fresh culture medium. We applied the same protocol to all samples.

We used a colorimetric assay, WST-1 based (Cell Proliferation Reagent WST-1, Roche Diagnostics GmbH, Mannheim, Germany) to assess the activity of the mitochondria at one hour, twenty-four hours as well as seven days, the same protocol which is described by Birkenmaier et al. and Ficklscherer et al. (61, 63). We changed the medium to alpha-MEM containing 10% WST-1 reagent and then returned the samples to the incubator for an additional two hours. In this test, WST-1, which is a tetrazolium salt, is cleaved into orange formazan by dehydrogenase enzyme found in mitochondria of viable cells. Increased mitochondrial activity increases the production of orange formazan; this will be quantified and measured with the assistance of an ELISA-reader (MWG-Biotech AG, Ebersberg, Germany) at 450 nm wavelength and a reference wavelength of 690 nm (63).

Table 1: Number of cells in each well for assay of mitochondrial activity

Drug	**Time after drug exposure**		
	1 h	24 h	7d
Epinephrine	6 x 5000 cells	6x 5000 cells	6 x 5000 cells
Vasopressin	6 x 5000 cells	6 x 5000 cells	6 x 5000 cells
Medium (control)	6 x 5000 cells	6 x 5000 cells	6 x 5000 cells

5.4 RNA analysis

For the RNA analysis, we used the same concentrations for both drugs and medium as we used for the proliferation assay. The groups were exposed to either epinephrine or vasopressin 0.033% (1 ampule in 3 l 0.9% NaCl). We treated one subgroup with alpha-MEM-only, and this group served as the control (Table 2). The culture medium (alpha-MEM) was fully aspirated, and 4 ml of vasopressin or epinephrine in its corresponding concentration was added to each well; no medication was added to the wells of the control group (Table 2). Then the cell was placed in an incubator at 37°C with 5%CO_2 for forty minutes. Forty minutes later, we have removed the solution containing epinephrine, vasopressin, or culture medium only, and then a new medium was supplied to the cells. All of the samples have been treated with the same protocol. Then the culture medium was removed before washing the cells with PBS (Biochrom GmbH, Berlin, Germany). In the next step, cells were lysed with 1 ml of QIAzol Lysis Reagent (Qiagen GmbH, Hilden, Germany) and relocated into a 1.5 ml Eppendorf tube with safety lock. Next, 200 µl of chloroform (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) was added, then the samples were stacked and incubated about ten minutes under room temperature. After that, we have put the cells in the centrifuge at 15,000 x g at 4°C for twenty minutes. Aqueous-phase has been isolated from the centrifuged samples and was added into new tubes. 0.5 ml isopropanol (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) has been used to precipitate RNA, the samples were incubated about ten minutes under room temperature and then centrifuged at 15,000 x g at 4°C overnight. Ethanol 75% (Merck KGaA, Darmstadt, Germany) has been used for washing the RNA-pellets before centrifuging two times under room temperature for twenty minutes at 15,000 x g. Then the pallets have been dried. After that, we used 32 µl of RNaseFree distilled water (GIBCO Corp., Darmstadt, Germany) to solving RNA. The purity and concentration were checked with Nanodrop-1000 (ThermoFisher Scientific, Waltham, Massachusetts, USA).

Table 2: Number of cells in each well for RNA analysis

Drug	**Time after drug exposure**		
	1 h	24 h	7 d
Epinephrine	1 x 10^5 cells	1 x 10^5 cells	1 x 10^5 cells
Vasopressin	1 x 10^5 cells	1 x 10^5 cells	1 x 10^5 cells
Medium (control)	1 x 10^5 cells	1 x 10^5 cells	1 x 10^5 cells

5.5 Real-time PCR

By using of QuantiTect RT-kit (Qiagen GmbH, Hilden, Germany), which included DNAse treatment, one µg total RNA of each sample was reverse transcribed to synthesize cDNA. The LightCycler96 System (Roche Applied Science, Mannheim, Germany) was used for polymerase chain reaction (PCR). The following primer pairs have been used for GAPDH (glyceraldehyde 3-phosphate dehydrogenase): primer sense, 5'-TGC ACC AAC TGC TTA GC-3', primer antisense, 5'-GGC ATG GAC TGT GGT CAT GAG-3' (64). The following primers pairs have been used for apoptosis specific proteins: CASP3 (caspase-3): primer sense, 5'-TGA AGC TAC CTC AAA CTT CC-3', primer antisense, 5'-CAG CAT CAC TGT AAC TTG CT-3' (65), and for PARP-1: primer sense, 5'-CCC AGG GTC TTC GGA TAG-3', primer antisense: 5'-AGC GTG CTT CAG TTC ATA CA-3' (66). To perform the amplification reactions we have used 2.5 µl of cDNA 1 to 3 diluted, 5 µl of FastStart Essential DNA Green Master (Roche

Applied Science, Mannheim, Germany), and 0.5 µl (500 nM) of primer pairs in 10 µL of final-volume. To performing PCR for GAPDH and PARP-1 the following protocol was used: at 95°C for 10 min, then 40 cycles at 95°C for 10s, at 60°C for 10s, and at 72°C for 15s. To performing PCR for CASP3 the following protocol was used: at 95°C for 10 min, then 40 cycles at 95°C for 10s, at 63°C for 10s, and at 72°C for 15s. We performed the reactions in triplicate. We calculated the relative quantification values of the target genes CASP3 and PARP-1 using the delta-delta Ct method normalized to GAPDH.

5.6 Statistical data analysis

We analyzed our data by using one-way ANOVA (one-way analysis of variance), which was followed by the t-test Bonferroni (Prism, GraphPad Software Inc., San Diego, California) in order to compare between the viability of the cells and apoptosis markers statistically. We have considered a probability of $p < 0.05$ as significant.

6 Results

6.1 Assessment of mitochondria activity

The activity of mitochondria in hMSCs exposed to epinephrine or vasopressin for forty minutes was not significantly lower than the activity of cells in the control group when measured one hour, twenty-four hours and seven days after exposure (Figure 10).

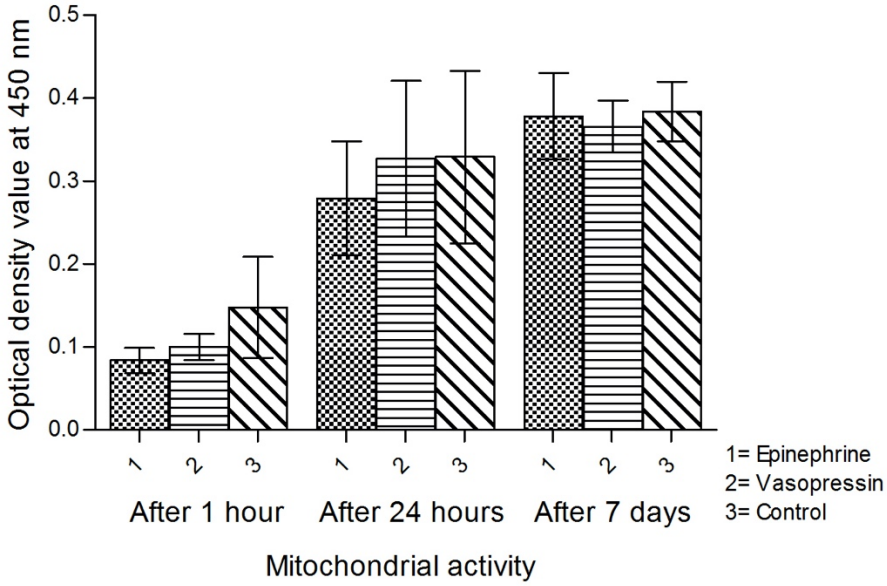

Figure 10. Bar chart (with standard deviation) of mitochondrial activity of hMSCs according to time after exposure to epinephrine or vasopressin. No time-dependent impact on the activity of the mitochondria was noted after forty minutes of exposure to vasopressin or epinephrine. No significant differences in the activity of mitochondria were noted one hour, twenty-four hours, and seven days after hMSCs were exposed to vasopressin for forty minutes.

6.2 Apoptosis rate

To assess apoptosis, we measured levels of PARP-1 (Figure 11 to 13) and CASP3 (Figure 14 to 16) at one hour, twenty-four hours, and seven days. In our experiment, we could find a significant ($p < 0.05$ - 0.001) higher amount of PARP-1 in hMSCs twenty-four hours and seven days after exposure to vasopressin (Figures 12 and 13) and seven days after exposure to epinephrine (Figure 13).

The amount of CASP3 increased adequately ($p < 0.001$) only in the group of samples, which was exposure to epinephrine just for twenty-four hours (Figure 15).

There was no significant higher level of PARP-1 after one hour of exposure to epinephrine or vasopressin (Figure 11). There was no significant higher level of CASP3 after one hour, twenty-four hours, or seven days of exposure to vasopressin (figure 14 to 16). There was no significant higher level of CASP3 after one hour or seven days of exposure to epinephrine (Figures 14 and 16).

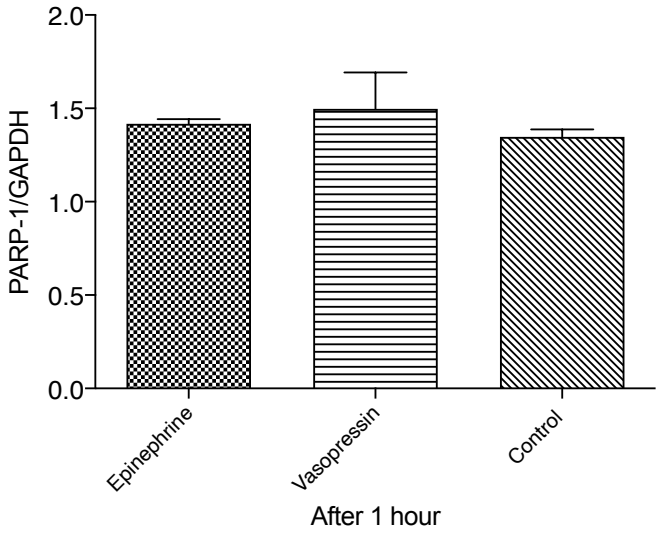

Figure 11. Bar chart (with standard deviation) of PARP-1 levels in hMSCs after one hour of epinephrine or vasopressin exposure. No significant elevation of PARP-1 was noted one hour after hMSCs were exposed to epinephrine or vasopressin for forty minutes.

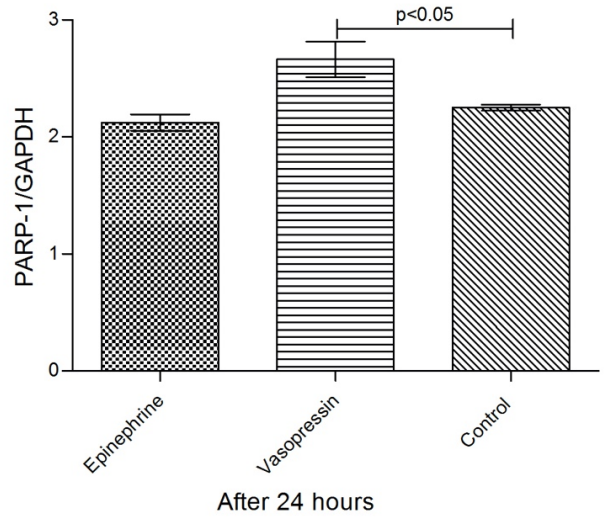

Figure 12. Bar chart (with standard deviation) of PARP-1 levels in hMSCs after twenty-four hours of epinephrine or vasopressin exposure. A significant elevation of PARP-1 was only noted twenty-four hours after hMSCs were exposed to vasopressin for forty minutes.

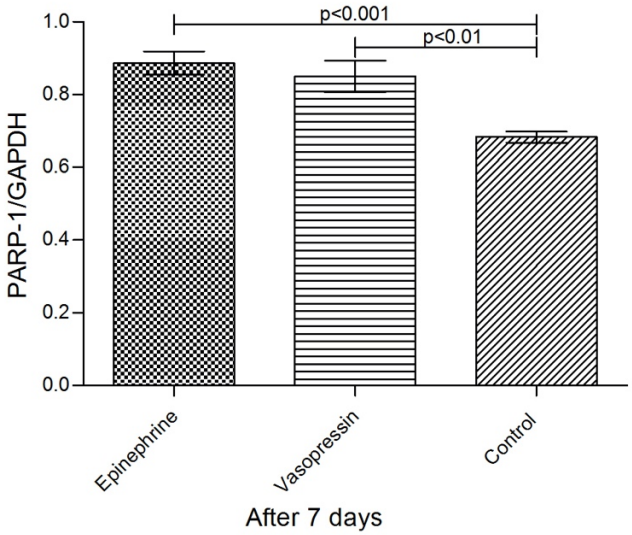

Figure 13. Bar chart (with standard deviation) of PARP-1 hMSCs after seven days of epinephrine or vasopressin exposure. A significant elevation of PARP-1 was noted seven days after hMSCs were exposed to epinephrine and vasopressin for forty minutes.

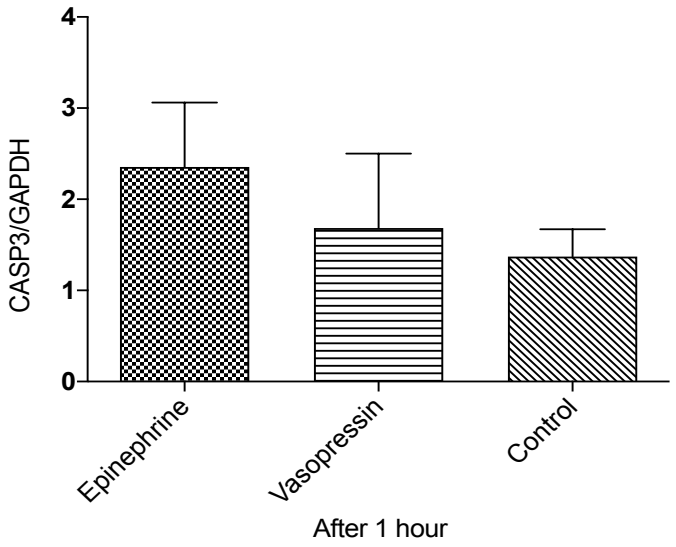

Figure 14. Bar chart (with standard deviation) of CASP3 levels in hMSCs after one hour of epinephrine or vasopressin exposure. No significant elevation of CASP3 was noted one hour after hMSCs were exposed to epinephrine and vasopressin for forty minutes.

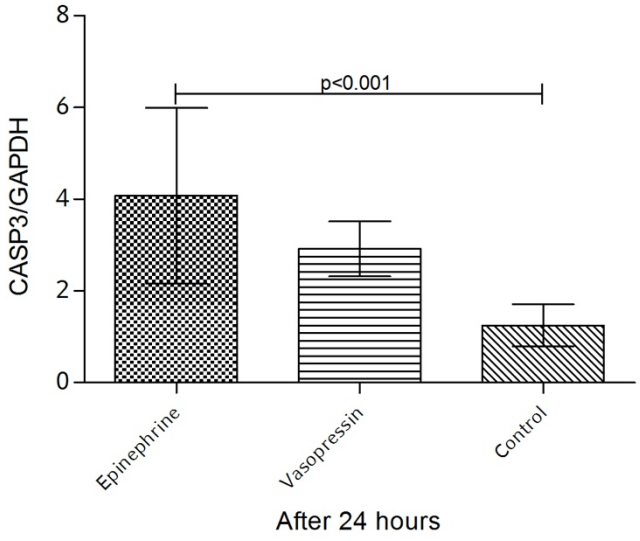

Figure 15. Bar chart (with standard deviation) of CASP3 levels in hMSCs after twenty-four hours of epinephrine or vasopressin exposure. A significant elevation of caspase-3 was noted only twenty-four hours after hMSCs were exposed to epinephrine for forty minutes.

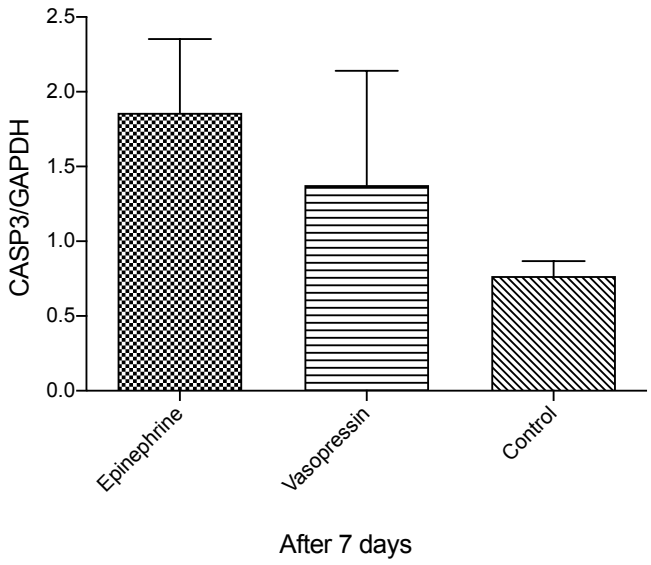

Figure 16. Bar chart (with standard deviation) of CASP3 levels in hMSCs after seven days of epinephrine or vasopressin exposure. No significant elevation of CASP3 was noted seven days after hMSCs were exposed to epinephrine or vasopressin for forty minutes.

7 Discussion

Controlling intraoperative bleeding during knee or shoulder arthroscopic procedures is challenging. Intra-articular irrigation with local hemostatic agents has repeatedly demonstrated safe and effective control of intraoperative bleeding in arthroscopy (48-50). Previous studies have evaluated the impact of local hemostasis on the visualization of the surgical field (48-50) or the cytotoxicity of local anesthetics on hMSC, fibroblasts, or chondrocytes (58-62, 67). This investigation is the first one to examine the effects of epinephrine and vasopressin on hMSCs to see if these medications have any adverse effects.

The wide range of tissues in which they reside, pluripotent potential, and mesoderm origin make hMSCs essential players in the field of regenerative medicine (9). hMSCs are essential in natural tissue regeneration processes: during the proliferative and remodeling phases, they migrate to the defected area and differentiate to replace cartilage, bone, or other tissues (68). Human mesenchymal stem cells have garnered considerable attention among the methods used for regenerating tissues, especially in cartilage regeneration. Because of the critical role of these cells in the complex process of cartilage reconstruction, we aimed to examine and evaluate whether common hemostatic agents used intra-articularly affect hMSCs and whether they induce apoptosis. Until now, investigations have mainly concentrated on the efficacy of these medications for hemostasis and their effects on the operative visual field. We chose epinephrine as the hemostatic agent because it has shown the improvement in operative field clearance in previous studies and is commonly used during arthroscopy (48-50). Vasopressin has been used as a vasopressor with positive outcomes (53-57), but until now, it has never been used in arthroscopy, and it has never been evaluated for its potential toxicity to hMSCs.

Our most important finding is that neither epinephrine nor vasopressin has detrimental effects on the proliferation of hMSCs as determined by proliferation assays and mitochondrial activity. However, the cells did show increasing apoptosis over time after exposure to the drugs. We quantified mitochondrial activity to determine stem cell viability (69 and 70). We measured expression levels of apoptosis markers CASP3 and PARP-1 to evaluate drug-induced apoptosis (59, 61, and 62). We did not note any significant decreases in mitochondrial activity in hMSCs exposed to vasopressin or epinephrine compared to activity in the control group at any time point. It may be because of the tremendous potential of MSCs self-renewal (10, and 11). PARP-1 was increased significantly, twenty-four hours, and seven days after exposure to vasopressin (Figure 12 and 13) and seven days after exposure to epinephrine (Figure 13). CASP3 significantly increased only twenty-four hours after exposure to

epinephrine (Figure 15), but CASP3 did not show additional increases seven days after epinephrine exposure (Figure 16). This can be explained by changes in intracellular calcium dysregulation that lead to caspase-independent mechanisms of cell death (71-74). However, there was no significant change in CASP3 after exposure to vasopressin. We believe that there is an increase in the apoptosis rate of the hMSCs due to an increase in CASP3 and PARP-1 after exposure to epinephrine, but the potential of hMSC self-renewal offsets this effect during the period of the time. So we are recommending not to use this medication in cases such as a microfracture, ACI, OATS, or even after the tendon/meniscal repair. As we see just an increase in PARP-1 after exposure to vasopressin without any increase in CASP3, we propose that it could be less toxic for the hMSCs and maybe an excellent alternative to epinephrine, but the further investigation should be done to see if this vasopressin can also improve the visual field during arthroscopy.

The results of our study demonstrate that forty minutes of exposure to either vasopressin or epinephrine in vitro does not decrease stem cell mitochondrial activity. PARP-1 was significantly higher in those cells which were treated by vasopressin for twenty-four hours and seven days; PARP-1 increased significantly only in the group of cells after seven days of epinephrine exposure. In comparison, Fickelscherer et al. demonstrated that 0.05 percent, 0.025 percent, and 0.0125 percent of fentanyl were able to decrease the proliferation of human mesenchymal stem cells (hMSCs). However, only 0.05 a percent fentanyl dilution could start apoptosis in human mesenchymal stem cells (hMSCs), as shown by a significant increase in CASP-3 (59).

7.1 Limitations

There are some limitations to our study that must be considered. First, this study is an in vitro experiment, and this model does not demonstrate the same condition as an intraoperative and intra-articular status. Confounding conditions may include blood supply, metabolic activities of the cells, different amounts of synovial fluid, and active bleeding (61). Also, since this was an in vitro experiment, our perceptions cannot be extrapolated to in vivo conditions. However, in vitro investigations that are performing under standardized conditions allow for the reproducibility of results related to metabolic activity, induction of apoptosis, and impacts on cell viability that depends on the substance. Additional researches and experiments should be perfumed to evaluate the effects of epinephrine and vasopressin on hMSCs in intra-articular conditions. The effects of these medications closer to the cell membrane should be evaluated in future studies. Accurately, the inducible nitric oxide synthase expression, which rapidly synthesizes large quantities of nitric oxide (a free radical), could be determined. Overproduction of nitric oxide causes damage to cellular components such as lipids, which decreases physiological function and leads to cell death (61, 75, and 76). Additionally, our results require to be confirmed on animal models.

7.2 Conclusion

Our conclusion based on our findings is that epinephrine and vasopressin have shown varying effects on apoptosis in hMSCs in vitro, depending on the time without any effect of the cell proliferation. Because of the increase in the apoptosis rate without the decrease in mitochondrial activity, the levels of toxicity remain unclear. The absence of a response of CASP3 after exposure to the vasopressin, suggest that vasopressin may be less toxic for hMSCs. Furthermore, it´s worth mentioning again that this is an in vitro investigation. Further investigations are required in order to evaluate the effects of

these two medications on hMSCs and translate the outcome into clinical practice. Our experiment as an in vitro model suggests that both epinephrine and vasopressin are potentially toxic drugs for intra-articular application during arthroscopic procedures such as microfracture, ACI, OATS (any kind of cartilage-repair), and a tendon or ligament reconstruction.

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