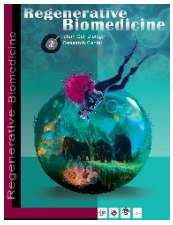

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Review Article

Therapeutic Potential of Amniotic Fluid and Amniotic Fluid-Derived Stem Cells in Regenerative Medicine

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Abstract

Amniotic fluid (AF) serves as a protective medium for the developing fetus and is a rich source of biologically active components, including fetal stem cells and extracellular vesicles (EVs). Notably, mesenchymal stem cells (MSCs) and human amniotic epithelial cells (hAECs) in AF possess pluripotent-like characteristics, contributing to the development of therapeutic strategies.

This review aims to explore the therapeutic potential of AF-derived stem cells (AFSCs) in regenerative medicine and immunotherapy.

The review consolidates findings on the Mesenchymal Stem Cells (MSCs) and human amniotic epithelial cells (hAECs) stand out for their pluripotent-like characteristics, with hAECs originating from epiblast-derived stem cells. AF-MSCs offer several advantages over adult MSCs, addressing ethical concerns and showcasing therapeutic potential.

The distinctive biological attributes of AF, including its components and derived cells, make it a promising candidate for therapeutic applications in regenerative medicine and immunotherapy strategies. Further research is warranted to elucidate specific clinical applications and mechanisms underlying its beneficial properties.

Keywords: Amniotic Fluid Mesenchymal Stem Cells, Cardiovascular diseases, Infertility, Neurodegenerative disease, Regenerative Medicine, Wound healing

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Introduction

Amniotic fluid (AF) and the stem cells derived from it hold immense promise in regenerative medicine and therapeutic applications. This unique biological medium, which surrounds and protects the developing fetus, provides essential mechanical and biochemical support and serves as a reservoir of diverse bioactive molecules and potent stem cell populations (1). The composition of AF evolves with gestational age, resulting in an increasing number and diversity of amniocytes. These amniocytes—a collective term for all cells in AF—include differentiated cells, progenitor cells, and stem cells of varying potency. This dynamic cellular composition underscores AF's unique potential in regenerative medicine (2).

Among these are mesenchymal stem cells (MSCs), hematopoietic progenitors, and multipotent stem-like cells, all of which exhibit significant regenerative potential and versatility (3, 4). Derived from the cellularly diverse AF, which forms early in gestation and evolves throughout pregnancy, amniotic fluid MSCs (AF-MSCs), offer distinct advantages over adult stem cells, including higher differentiation potential and minimal epigenetic alterations (5). AF contains various cell types, including differentiated cells, progenitors, and multipotent stem cells, enriched by its proximity to fetal compartments. Among these, AF-MSCs stand out for their ability to differentiate into all three germ layers, high proliferative capacity, and regenerative potential (6).

Recent advancements in the characterization and isolation of AF-derived stem cells have revealed their capacity for differentiation into multiple lineages, making them a powerful tool for tissue engineering, disease modeling, and cell-based therapies (7). Furthermore, the accessibility and non-invasive collection methods of AF underscore its practicality as a source of therapeutic agents. This paper explores the composition, biological properties, and therapeutic potential of amniotic fluid stem cells, emphasizing their role in advancing modern medicine.

Amniotic fluid products

Research into AF-derived cells began in the late 1960s, with an initial emphasis on optimizing cultivation techniques and culture conditions (2). A breakthrough occurred in 1993 when Torricelli et al. identified hematopoietic progenitor cells in AF, underscoring its potential as a regenerative cell source (8). AF-MSCs surpass adult MSCs in ethical suitability and therapeutic potential, offering superior clonogenicity, differentiation capacity, and plasticity (9-11). They also display faster growth rates and longer telomeres, attributable to higher telomerase activity (12). AF surrounds various developing fetal tissues and contains a mixture of fetal cells and human amniotic epithelial cells (hAECs) released from the amnion into the fluid. Since the amnion arises directly from the epiblast layer, AF is thought to harbor pluripotent stem cells derived from epiblast stem cells during the pre-gastrulation phase (13, 14).

AF is rich in clonal amniocytes and their secretome, which includes extracellular vesicles (EVs) and exosomes. These substances can be extracted from the acellular fraction of AF or derived from cultured AF-MSCs, yielding products referred to as AF-MSC-EVs and AF-MSC-Exo, respectively. Figure 1 illustrates the process of obtaining

these materials from residual second-trimester human AF (hAF) and AF-MSCs, which are cultivated from primary cultures during prenatal diagnostic amniocentesis, as detailed in our recent study (7).

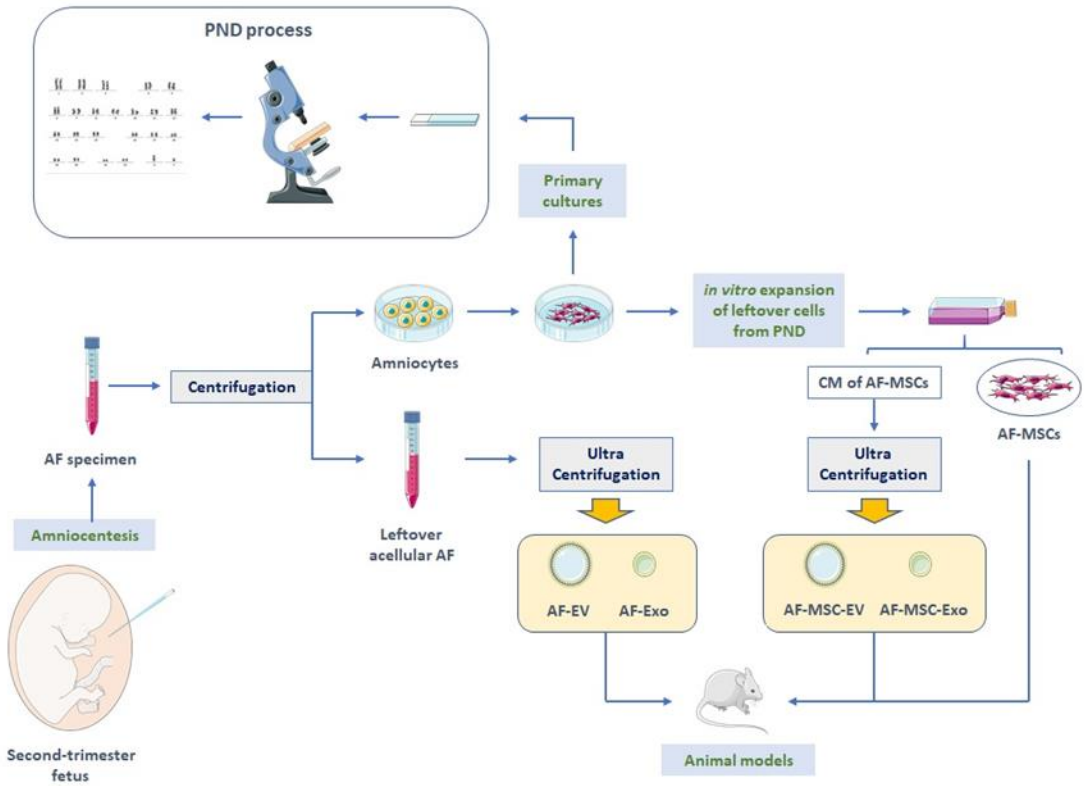

Figure 1. Acquisition of amniotic products for clinical applications from leftover second-trimester human amniotic fluid during prenatal diagnostic amniocentesis. Abbreviations) hAF: human amniotic fluid; AF-MSCs: amniotic fluid mesenchymal stem cells; CM: conditioned medium; PND: prenatal diagnosis; EV: extracellular vesicle; Exo: exosome.

immunogenicity and their repertoire of immunomodulatory molecules. These properties enable them to effectively suppress immune responses (15). Clinical studies have extensively demonstrated the regenerative potential of AF-MSCs, particularly in the repair of various tissues such as cardiac tissue, bone, and cartilage (16). Furthermore, numerous investigations have underscored

Clinical application of amniotic fluid stem cells

AF-MSCs and their clinical products, including EVs (AF-MSC-EVs) and exosomes (AF-MSC-Exo), offer significant advantages in medical applications due to their low

their positive impact on healing internal injuries, including those affecting the bladder, lungs, and kidneys. They also exhibit promising potential in treating neurodegenerative diseases (17).

Innovative therapeutic applications for AF-MSCs are emerging, such as their use in treating hemophilia A. A recent study demonstrated the efficacy of prenatal intrahepatic AF-MSC administration in mice models (18). Additionally, these cells have shown promise in addressing congenital abnormalities (19). Comparative studies

reveal that AF-MSCs produce a higher quantity of exosomes than bone marrow-derived MSCs, further enhancing their therapeutic value (20). Notably, while AF-MSC-EVs contain microRNAs (miRNAs), they lack mRNAs, suggesting a direct or indirect role in modulating pre-existing signaling pathways (15).

In recent years, many studies have been conducted on different ways of using AF-MSCs and their clinical products, AF-MSC-EVs and AF-MSC-Exo, in an attempt to treat, reverse or even prevent many diseases and conditions. Herein, this review explores the latest advancements in the application of AF-MSCs across diverse medical fields, highlighting their transformative potential in regenerative medicine and innovative therapies.

Cardiovascular diseases

Cardiovascular diseases (CVDs) remain a leading cause of morbidity and mortality worldwide. Despite significant advancements in conventional treatments such as pharmacotherapy, percutaneous interventions, and surgical procedures, the capacity for complete cardiac repair and regeneration remains limited. Innovative approaches, including regenerative medicine, have emerged as promising alternatives. Among these, stem cell-based therapies have gained substantial attention for their potential to repair and regenerate damaged cardiac tissue (21).

AF-MSCs present a unique and promising source of stem cells for cardiac regeneration due to their immunomodulatory properties, low tumorigenic risk, and inherent ability to differentiate into cardiomyocytes. Additionally, AF-MSCs secrete various bioactive molecules, including growth factors and cytokines, that contribute to tissue repair, angiogenesis, and anti-fibrotic effects (22, 23). These properties underline the immense therapeutic potential of AF-MSCs in addressing the unmet needs in CVD treatment (24).

Despite the encouraging results, the precise mechanisms underlying the therapeutic effects of AF-MSCs in cardiovascular applications remain incompletely understood. Emerging evidence suggests that the therapeutic efficacy of AF-MSCs is largely mediated through paracrine signaling rather than direct differentiation (25). Exosomes and EVs derived from AF-MSCs are increasingly recognized as critical mediators of these paracrine effects, offering a potential cell-free therapeutic approach (15). In a study, injecting AF-MSC-EVs improved cardiac regeneration via paracrine modulation of endogenous mechanisms. By enhancing cardiac function, AF-MSC-EVs can potentially be seen as an appealing future pharmacotherapeutic agent (23, 26). A study on EVs derived from hAF-MSCs (AF-MSC-EV) reported these EVs are specifically enriched with miRNAs over proteins or other soluble factors; notably direct trafficking of miR-210 and miR-199a-3p from AF-MSC-EV to responder cells were suggested to drive pro-survival and proliferative effects in recipient human dermal fibroblast and murine myoblasts, which showed significant increase of such putative molecular candidates (27). Moreover, it has been revealed that AF-MSC-Exo can enhance angiogenesis in endothelial cells. Along with their anti-apoptotic properties and debilitating cardiac fibrosis, they can be a

novel promising approach for repair and regeneration of the heart (28).

Ischemic heart disease and myocardial infarction

Ischemic heart disease (IHD), also called coronary artery disease (CAD), refers to a group of conditions caused by reduced blood flow to the heart muscle due to partial or complete blockage of the coronary arteries, while myocardial infarction (MI), commonly known as a heart attack, is an acute and severe form of IHD that occurs when blood flow to a part of the heart muscle is completely blocked, leading to damage or death of heart muscle tissue. MI is characterized by irreversible damage to cardiac tissue due to prolonged ischemia, often leading to heart failure if untreated (29).

AF-MSCs mitigate ischemia-induced cardiomyocyte apoptosis through their anti-inflammatory and antioxidant properties, leading to improved clinical outcomes in IHD models (30). Studies have demonstrated that AF-MSCs can significantly improve cardiac function and reduce infarct size in preclinical MI models. AF-MSCs promote angiogenesis through the secretion of pro-angiogenic factors, such as VEGF and FGF, thereby enhancing blood supply to the ischemic myocardium (31).

Bollini et al., investigated activation of the myocardial gene program in rat c-kit+ AFSCs which was induced by co-culture with neonatal rat cardiomyocytes (rCMs). The in vivo potential of c-kit+ AFSCs for myocardial repair was studied by transplantation in the heart of animals with ischemia/reperfusion injury, monitored by magnetic resonance imaging (MRI). Three weeks after injection a small number of AFSCs acquired an endothelial or smooth muscle phenotype and to a lesser extent CMs. Despite the low AFSCs count in the heart, there was still an improvement of ejection fraction as measured by MRI. their finding showed that AFSCs have the in vitro propensity to acquire a cardiomyogenic phenotype and to preserve cardiac function, even if their potential may be limited by poor survival in an allogeneic setting (32).

AF-MSCs have been shown to improve cardiac function and reduce infarct size in MI models. They promote the formation of new blood vessels and reduce scar tissue formation. Furthermore, they reduce scar tissue formation by modulating inflammatory responses and enhancing endogenous cardiac repair mechanisms (31). Balbi et al. investigated the therapeutic potential of the hAFSC secretome for cardiac regeneration. Their study examined the anti-apoptotic, angiogenic, and proliferative effects of hAFSC-conditioned medium (hAFSC-CM) on rodent neonatal cardiomyocytes (rNCMs), human endothelial colony-forming cells (hECFCs), and human cardiac progenitor cells (hCPCs). In a myocardial infarction (MI) mouse model, they administered a single intra-myocardial injection of hAFSC-CM, EVs (hAFSC-EVs), or EV-depleted conditioned medium (hAFSC-DM) and assessed outcomes over short- and long-term periods. The findings revealed that hAFSC-CM significantly enhanced cardiomyocyte survival, angiogenesis, and cell proliferation, leading to reduced scarring and improved cardiac function and regeneration. While hAFSC-EVs replicated most of hAFSC-CM's therapeutic benefits—except for angiogenesis—hAFSC-DM showed no

efficacy. Overall, the hAFSC secretome supports sustained cardiac repair by modulating endogenous regenerative mechanisms. Notably, hAFSC-CM demonstrated superior pro-angiogenic effects, whereas hAFSC-EVs were more effective in improving cardiac function. Both approaches facilitate myocardial renewal and regeneration after injury (23).

Wang et al., investigated the effects of transplanting AF-MSCs genetically modified to overexpress the Akt gene into ischemic rabbit myocardium. After three weeks, histological analysis showed reduced myocardial inflammation and structural damage, increased capillary density, and higher levels of proteins like GATA4, connexin 43, and cardiac troponin T in the Akt-AFMSC group compared to controls. Additionally, there was a decrease in cardiomyocyte apoptosis, with increased levels of phosphorylated Akt and Bcl-2 and reduced caspase-3 activity. Improved left ventricular function was also observed. These findings suggest that Akt-modified AF-MSCs provide protection by promoting angiogenesis, preventing cell death, and potentially differentiating into cardiomyocytes, which enhance their survival and therapeutic benefits. Overall, this approach alleviates ischemia-reperfusion (I/R) injury and improves heart function (33). In addition, AF-MSC-Exo prevented rat hearts from getting ischemia–reperfusion injury in vivo, showed cardioprotective and marked promigratory abilities which can be used in treating myocardial infarction (34).

Cardiac remodeling and heart failure

Adverse cardiac remodeling is defined as structural and functional changes in the myocardium following acute and/or chronic cardiac injury. Cardiac remodelling after acute MI is the main cause of the heart failure (HF). HF, a condition often resulting from MI or chronic cardiac stress, is characterized by impaired myocardial contractility and progressive fibrosis (35). AF-MSCs have been shown to attenuate cardiomyocyte apoptosis and ventricular remodeling, as well as strongly promote capillary formation at the infarct border zone by modulating extracellular matrix (ECM) deposition and reducing pathological fibrosis (31). Recent studies, for instance on pulmonary fibrosis, which is characterized by excessive extracellular matrix production and deposition, have shown that AF-MSCs secrete matrix metalloproteinases (MMPs) led to maintaining ECM homeostasis and preventing excessive fibrotic tissue deposition (36).

Recent studies have shown that AF-MSCs can improve heart function in heart failure conditions and enhance myocardial repair by reducing fibrosis and promoting the survival of existing cardiomyocytes (37). Preclinical studies indicate that AF-MSC transplantation improves left ventricular ejection fraction (LVEF) and reduces ventricular dilation, highlighting their potential in reversing cardiac dysfunction (33, 38). The paracrine effects of AF-MSCs, including the release of anti-apoptotic and pro-regenerative factors, are critical in mediating these benefits.

A recent study explored the therapeutic potential of exosomes derived from human amniotic fluid mesenchymal stem cells (hAF-MSCs, or AF-Exos). These exosomes were

found to reduce cardiac fibrosis by promoting angiogenesis in human umbilical vein endothelial cells (HUVECs) after oxygen and glucose deprivation (OGD) in vitro and in isoproterenol (ISO)-induced cardiac fibrosis in vivo. The in vitro findings demonstrated that AF-Exos enhanced HUVEC motility, migration, and tube formation post-OGD, significantly increasing total tube length, branching points, and loops compared to phosphate-buffered saline (PBS). Furthermore, in vivo findings demonstrated that AF-Exos reduced cardiac fibrosis severity based on decreased levels of fibrosis markers (Collagen I and α-smooth muscle actin). Microvessel density analysis also confirmed more regenerated microvessels in AF-Exos-treated rats. Moreover, elevated hypoxia-inducible factor 1α (HIF-1α) and vascular endothelial growth factor (VEGF) levels were observed in treated left ventricles. In conclusion, these results suggest AF-Exos has proangiogenic effects and could serve as a promising strategy for treating cardiac fibrosis (28).

Fibrosis results from the excessive deposition of extracellular matrix (ECM) components essential for injury repair. The molecular pathology of fibrotic diseases varies, involving multiple bioactive factors in the pathogenic process. Recent insights into fibrotic pathogenesis suggest that resident MSCs may differentiate into myofibroblasts, contributing to fibrosis progression (39). Conversely, preclinical and clinical studies on autologous or allogeneic MSC infusions have shown that MSCs can alleviate fibrotic diseases by modulating inflammation, regenerating damaged tissues, remodeling ECM, and mitigating stress-induced cell death. This dual role of resident and exogenous MSCs in fibrotic diseases remains a topic of significant debate (40).

Furthermore, despite the optimistic results regarding the regenerative properties of AF-MSCs, some reports challenge this idea. For example, a study found that AFSCs do not successfully differentiate into functional cardiomyocytes using a well-established Wnt signaling modulation protocol. Despite expressing cardiac-specific genes and proteins, differentiated AFSCs lacked spontaneous contractility and showed diminished ion channel activity compared to hESC-derived cardiomyocytes. These findings suggest that AFSCs may not be suitable for cardiomyocyte differentiation under the tested conditions (41).

Wound healing

More than 40 years ago, Rowlatt (42) observed the absence of scar formation in 20-week-old human fetal skin after trauma. Since then, significant research has explored scar formation mechanisms. Fetal scar-less healing involves shorter inflammation periods, fewer activated macrophages, enhanced fibroblast migration, increased hyaluronic acid receptor expression, and distinct growth factor profiles compared to adult wound healing (43, 44). Fetal dermal fibroblasts synthesize more type III collagen and form a lattice that promotes dermal regeneration while minimizing fibrosis (45). Based on these findings, many studies have been designed to explore the efficiency of fetal stem cells in wound healing. Accordingly, recent studies highlight the unique ability of AF-MSCs to accelerate wound healing and reduce scarring (43, 46).

AF-MSCs can play a significant role in wound generation especially in scar-free wound healing after injury through reducing fibrotic scarring and promoting neovascularization due to their paracrine secretions in favour of regulating the microenvironment and regeneration of immune homeostasis (47, 48). A study examined the paracrine factors released by hAF-MSCs and their ability to accelerate the wound-healing process by stimulating proliferation and migration of dermal fibroblasts. AF-MSC-conditioned media (AF-MSC-CM) significantly enhanced the proliferation of dermal fibroblasts. Their findings from protein-based assays indicated that AF-MSC-CM contains various cytokines and chemokines that are known to be important in normal wound healing, including IL-8, IL-6, TGF-beta, TNFRI, VEGF, and EGF (49).

Aberrant scar formation is associated with a pathological disorganized wound-healing process with chronic inflammation. The TGF-β/Smad signaling pathway is the most canonical pathway through which the formation of collagen in the fibroblasts and myofibroblasts is regulated. Sustained activation of the TGF-β/Smad signaling pathway results in the long-term overactivation of fibroblasts and myofibroblasts, which is necessary for the excessive collagen formation in aberrant scars. Therefore, therapeutic strategies aim to target the TGF-β/Smad signaling pathway in fibroblasts and myofibroblasts to interfere with their cellular functions and reduce cell proliferation (50). Accordingly, cell therapy approaches consider TGF-β/Smad signaling pathway for scar-free wound healing. For example, Yoon et al. demonstrated that application of AF-MSC-CM significantly enhanced wound healing by dermal fibroblasts via the TGF-β/SMAD2 pathway. Their findings revealed that levels of SMAD2 were increased by AF-MSC-CM, and both the increase in SMAD2 and migration of dermal fibroblasts were blocked by inhibiting the TGF-β/SMAD2 pathway. Moreover, in a mouse excisional wound model, AF-MSC-CM accelerated wound healing. These data provide the first evidence of the potential for AF-MSC-CM in the treatment of skin wounds (49).

AFSCs in keratinocyte-conditioned media promote wound closure by differentiating into keratinocytes (51), secreting paracrine signals via the TGF-β/SMAD2 pathway, and enhancing ECM regulation with anti-fibrosis mediators. This reduces type I collagen bundles, increases type III collagen, and spares the granulation tissue area (51, 52). Fukutake et al. (51) demonstrated that injecting 1×10^6 c-KIT$^+$ hAFSCs into full-thickness 1 cm^2 skin defects led to their presence in the dermis for up to 7 days. This suppressed α-smooth muscle actin-positive myofibroblasts and enhanced type III collagen expression, improving ECM remodeling and wound closure without relying on contraction. Their results also revealed that c-KIT$^+$ hAFSCs promote re-epithelialization of the epidermis while maintaining granulation tissue integrity in the dermis.

The use of MSC sheets is a promising strategy for skin regeneration. Besides findings found the efficiency of dissociated hAFSCs to accelerate cutaneous wound healing with reduced fibrotic scarring, the use of hAFSCs in applications of cell sheet technology has

also been recently highlighted. For example, Ochiai et al., recently examined the in vivo efficacy of in vitro-cultured hAFSC sheets in wound healing in BALB/c mice. Although the hAFSC sheet contained abundant extracellular matrix molecules and expressed high levels of anti-fibrotic mediators, its grafting did not affect wound closure or the size of the granulation tissue area. In contrast, the organization of type I collagen bundles in the regenerated wound was markedly reduced, while the levels of type III collagen were increased after implantation of the hAFSC sheet. These results suggest that hAFSC sheets can exert anti-fibrotic properties without delaying wound closure (52).

Another strategy recently highlighted for wound healing is using the secretome of MSCs. It has been demonstrated that AF-MSC-Exo can enhance efficiency of wound healing and increase regeneration of hair follicles along with nerves and vessels. For example, using CM of Nanog overexpressing AF-MSCs, augmented the telogen-to-anagen transition in hair follicles and increased hair follicle density (53). Moreover, AF-MSC-Exo can improve proliferation in cutaneous cells and simultaneously suppress the over-piling of myofibroblasts. By exhibiting these properties and functions, AF-MSC-Exo can prove to be a deliberate tool to avoid fibrotic scars in wound healing through exosomal specific miRNAs (54, 55). In a mice model, AF-MSC-EVs (e.g., exosome) promoted wound closure as a result of improving the proliferation and migration of dermal fibroblasts by conditioned media enriched in VEGF and TGF- β1. This henceforth presents a novel potent agent in the treatment of wound healing, postoperative scars and tissue engineering (56, 57).

Infertility and reproductive disorders

MSCs have emerged as a promising therapeutic strategy for addressing infertility in both males and females (58). Their regenerative capabilities, immunomodulatory properties, and ability to differentiate into various cell types make them ideal candidates for treating reproductive system dysfunctions (59, 60). Through their regenerative effects and participation in various paracrine pathways, MSCs have been shown to improve fertility outcomes (60).

Research has demonstrated that MSCs can differentiate into germ-like cells under specific induction conditions and contribute to gonadal tissue repair through transplantation. Both in vitro studies and animal model evaluations support the role of MSCs in promoting the recovery of spermatogenesis and folliculogenesis (58). A review of the literature reveals that most studies have focused on MSCs derived from bone marrow and umbilical cord, with relatively less emphasis on other MSC sources (58, 61). Notably, particular attention has been given to the therapeutic potential of AF-MSCs.

Female infertility

Disorders of the female reproductive system are associated with abnormalities in one or more the reproductive organs: ovaries, uterus, fallopian tubes, and cervix. These disorders can cause severe symptoms, including pain, frequent urination, altered menstruation, and are linked to negative reproductive outcomes, such as miscarriage and infertility (62). Reproductive disorders,

including intrauterine adhesion (IUA) (e.g., Asherman syndrome), premature ovarian insufficiency (POI), endometriosis, and polycystic ovary syndrome (PCOS), are great threats to female reproduction (63).

Several laboratory studies and clinical trials are investigating stem cells as a strategy for treating ovarian dysfunction and endometrial disorders that lead to infertility. In particular, several studies have focused on MSCs as an experimental approach to restoring ovarian function and treating infertility (64). Previously, the efficiency of autologous stem cell therapy has been confirmed in a clinical trial on 10 women suffering from idiopathic POI during that BM-MSCs was laparoscopically injected into the ovaries (65). POI is defined as a reduced function of ovaries before age 40 in which women may still have intermittent ovulation and occasional menstrual cycles, and in some cases, they may even conceive naturally (66). Several studies have reported that MSC transplantation improves ovarian function and ovarian reserve, and this action may be mediated by paracrine signaling pathways (67). However, previous studies have also suggested that the number of differentiated MSCs is not sufficient to account for the observed improvement in fertility, and controversy remains regarding the differentiation of MSCs into oocytes after migrating to target tissue (62).

AF-MSCs are being investigated for their therapeutic potential in treating female infertility caused by conditions such as PCOS, POI, and endometriosis (68). Studies in animal models have demonstrated promising outcomes, including the improvement of the ovarian microenvironment to restore normal ovarian activity, enhanced endometrial regeneration, and significant improvement in ovarian function at both physiological and molecular levels. Additional benefits reported include an increase in follicle counts and the restoration of hormonal balance in rat models (69, 70).

In vitro studies have shown that AFSCs can differentiate into oocyte-like cells, providing an optimal model for studying the mechanisms of oocyte development and maturation (71). Notably, AFSCs express germ cell markers such as DAZL (deleted in azoospermia-like gene) and exhibit the ability to differentiate into germ cell lineages, further highlighting their relevance in reproductive research (72). A study conducted by Asgari et al. compared the germline differentiation potential of MSCs derived from human amniotic tissue, chorionic tissue, and umbilical cords. The findings revealed that human chorionic MSCs exhibited the highest capacity for differentiation into female germline cells. Interestingly, umbilical cord MSCs demonstrated comparable differentiation potential into female germline cells, regardless of whether they originated from male or female umbilical cords (73). Further research by Alifi and Asgari (74) explored the use of retinoic acid (RA) as a key inducer for differentiating human amniotic membrane-derived MSCs (hAM-MSCs) into primordial germ cells (PGCs). Their study concluded that hAM-MSCs possess significant potential for germline differentiation and that RA serves as an effective inducer in this process. AF-MSCs can enhance folliculogenesis through paracrine signaling and the secretion of growth factors leading to promoting

angiogenesis and modulating oxidative stress (67). AF-MSCs also can rejuvenate ovarian function and increase ovarian reserve through the secretion of growth factors such as VEGF, HGF, and IGF-1, critical for follicular development and survival (69, 75). Additionally, AF-MSCs can be effective in resisting ovarian aging by resisting DNA damage by decreasing the expression of DNA damage markers (76). Moreover, AF-MSCs contribute to endometrial regeneration and improve implantation success rates in conditions like Asherman syndrome (75). Importantly, these cells modulate inflammation and fibrosis, key factors in uterine and ovarian pathologies, which can facilitate successful embryo implantation and pregnancy outcomes (69). AF-MSCs have also shown promise in improving ovarian reserve and restoring menstrual cycles in preclinical models (77, 78). Both hAF-MSCs and murine AF-MSCs (mAF-MSCs) have shown the ability to survive and proliferate in the ovary and to rescue short-term fertility of mice with chemotherapy-induced POF (premature ovarian failure implying a complete and irreversible cessation of ovarian function) after injection into the ovarian artery (77, 78). EVs have demonstrated therapeutic potential for female reproductive disorders by repairing injured endometrium, suppressing endometrial fibrosis, modulating immunity, exerting anti-inflammatory effects, and reducing apoptosis in ovarian granulosa cells. While the exact mechanisms underlying the effects of MSC-EVs are not fully elucidated, proposed pathways include the promotion of angiogenesis, regulation of immunity, and reduction of oxidative stress (63). Chemotherapy-induced POF remains a clinically irreversible and unfortunately common side effect of chemotherapy in women. However, significant progress has been made in mitigating this condition. For example, AF-MSC-Exo, enriched with microRNAs such as miR-146a and miR-10a, successfully prevented ovarian follicular atresia in chemotherapy-induced POF in mouse models. These exosomes represent a promising cell-free treatment, avoiding the complications associated with allogenic donor cells and unstable cell sources (Xiao et al. 2016). In addition, AF-MSC-Exo subsets have been shown to reduce apoptosis in ovarian granulosa cells, further underscoring their therapeutic potential in POF (79).

It has been reported that AECs, when injected into the tail vein of mice with POF, infiltrated damaged ovaries and facilitated the recovery of folliculogenesis, including differentiation toward granulosa cells (80, 81). Ding et al. extended these findings, demonstrating that both AECs and AF-MSCs restored the ovarian follicle pool across all developmental stages and normalized hormonal levels in chemotherapy-induced ovarian failure. Notably, while AECs caused less immunological rejection, AF-MSCs showed greater efficacy in restoring ovarian function, particularly in severe cases of POF (82). Innovative approaches to enhance the therapeutic effects of AF-MSCs have also been explored. Ling et al. demonstrated that pretreatment of human AF-MSCs with low-intensity pulsed ultrasound (LIPUS) significantly improved outcomes in POI rat models. Both pretreated and untreated AF-MSCs increased reproductive organ weights, reduced ovarian inflammation and granulosa cell apoptosis, and improved overall ovarian function (83).

AF-MSCs have also shown potential in ovarian cancer treatment. Studies indicate that AF-MSCs can reduce tumor size by 30% to 50%, transport therapeutic genes to tumor sites, and induce apoptosis via the secretion of IL-2 (84). Their natural tumor tropism enhances their utility in targeting ovarian tumors (85). Moreover, in cell models of high-grade serous ovarian cancer, AF-MSCs and their secretome, including exosomes, exhibited potent anti-tumor effects, outperforming their counterparts derived from chorionic villi (86).

Male infertility

Male infertility is closely linked to disruptions in spermatogenesis, a complex process requiring the coordination of germ cells and somatic cells, such as Sertoli and Leydig cells. These somatic cells play crucial roles in nourishing and supporting germ cell development (87). Various factors can compromise spermatogenesis. For example, advancing age can induce germ cell apoptosis and lead to abnormal spermatogenesis (87, 88). Similarly, reductions in testicular size impair sperm production and negatively affect fertility (58). Maintaining DNA integrity is also critical, as its disruption can result in sperm with impaired motility, abnormal morphology, and reduced functionality (89). Moreover, gonadotoxic treatments, such as radiation and chemotherapy, damage the somatic cell environment, disrupt spermatogonial differentiation, and severely impair sperm production, further exacerbating male infertility (58, 89).

Recent findings suggest that MSCs hold great promise in addressing these challenges. MSCs can differentiate into germ-like cells under specific conditions or upon transplantation into the testis. They enhance the local microenvironment of spermatogenesis, reconstructing the spermatogenic process by secreting nutritional and paracrine factors (90). MSCs are being actively explored for treating spermatogenic disorders and testicular dysfunctions caused by oxidative stress, inflammation, or structural damage. Their paracrine effects support the restoration of testicular microenvironments, while their differentiation potential contributes to regenerating Sertoli and Leydig cells, essential for sperm production and hormonal balance (58, 61, 87). Broadly, cell-based therapies for male infertility can be divided into two categories: in vitro differentiation into germ cells or gametes and in vivo transplantation of stem cells into reproductive organs (61).

When transplanted into the testis, MSCs enhance the local microenvironment necessary for spermatogenesis. This restoration occurs through the secretion of bioactive factors that nourish spermatogonial stem cells (SSCs), promote germ cell differentiation, and support somatic cell function (91-93). Besides AF-MSCs, other sources of MSCs, including bone marrow (BM), adipose tissue (AT), umbilical cord (UC), and amniotic fluid (AF), have been extensively studied for their potential to generate male germ cells in vitro.

For example, UC-MSCs treated with RA, testosterone, and testicular cell-conditioned medium have shown high expression of male germ cell markers and proteins (91). Similarly, AT-MSCs transdifferentiated into male germ-like cells using rabbit Sertoli cells

expressed germ cell-specific markers (94, 95). BM-MSCs, particularly when cultured with bone morphogenetic protein-4 (BMP-4), have demonstrated superior differentiation into primordial germ cells compared to AT-MSCs (96).

These studies underscore the potential of MSCs from various sources in germ cell generation, with differentiation efficiencies influenced by the MSC source and induction conditions. Among the various MSC sources, AF-MSCs exhibit unique advantages due to their high proliferation rate, pluripotency, and immunomodulatory properties. Afsartala et al. demonstrated for the first time that mouse AM-MSCs could differentiate into male germ cells when treated with BMP4 and RA. These findings highlight the potential of AF-MSCs as a source for in vitro germ cell generation and cell-based infertility therapies (97).

In vivo studies have further solidified the role of AF-MSCs in restoring fertility. For instance, Qian et al. explored the fertility-protective effects of hAM-MSCs against busulfan-induced testicular toxicity. Their results demonstrated that hAM-MSC transplantation restored spermatogenesis, improved testosterone levels, and enhanced testicular weight, size, and semen parameters. Mechanistically, hAM-MSCs reduced oxidative damage, repressed cell apoptosis, and enhanced proliferation in both in vivo and in vitro models (98).

These effects are attributed to the secretion of factors such as vascular endothelial growth factor (VEGF), insulin-like growth factor (IGF), and miRNAs, which regulate differentiation, regeneration, and protection of injured tissues (61).

MSCs and their secretomes are being evaluated for conditions such as testicular ischemia/reperfusion (I/R) injury, non-obstructive azoospermia (NOA), and varicocele-associated infertility. Ramesh et al. demonstrated that hAM-MSC secreted factors restored sperm chromatin condensation, improved spermatogenesis, and enhanced the histomorphometric organization of seminiferous tubules in a mouse model of I/R injury (99). Similarly, MSC transplantation in NOA animal models resulted in successful allotransplantation or xenotransplantation into seminiferous tubules, inducing spermatogenesis through paracrine signaling and exosome-mediated mechanisms (100). Recent studies also highlight the role of hAF-MSCs in varicocele models, a leading cause of male infertility. Transplantation of hAF-MSCs restored testicular architecture and function, as well as fertility parameters, by enhancing the expression of Sertoli cell markers and SOX9, a critical regulator of testicular development (101).

Musculoskeletal disorders

Musculoskeletal conditions encompass a wide range of disorders affecting joints, bones, and muscles. These include conditions such as osteoarthritis, psoriatic arthritis, and spondyloarthritis (joints); osteoporosis, osteopenia, fragility fractures, and traumatic fractures (bones); and sarcopenia (muscles) (102). Numerous studies have investigated the therapeutic potential of AF-MSCs in various conditions, including osteoporosis, osteopenia, periodontitis, maxillary sinus floor elevation (MSFE), and bone defects (103). One recent study highlights the role of AF-MSCs in vascular modeling of engineered

bone, though the exact mechanisms remain unclear (104). Furthermore, exosomes derived from MSCs have emerged as a promising therapeutic strategy for treating bone- and joint-associated musculoskeletal disorders. Studies demonstrate that MSC-derived exosomes can promote bone and cartilage recovery through mechanisms such as inhibiting inflammation, inducing angiogenesis, stimulating osteoblast and chondrocyte proliferation and migration, and negatively regulating matrix-degrading enzymes (105).

AF-MSC-EVs, particularly those containing miRNA, can modulate signaling pathways to promote tissue regeneration. For example, in a cardiotoxin-induced muscle injury model, hAF-MSC-EVs enhanced muscle regeneration through angiogenesis and inflammation regulation. These EVs increased the size of regenerating muscle fibers and the number of capillaries (26, 106). In another model of skeletal muscle atrophy, EVs derived from c-KIT$^+$ hAFSC were shown to reduce muscle inflammation in vivo (27).

Osteoporosis, characterized by reduced bone quality and increased fracture risk, is a significant area of research for AF-MSCs. Current treatments focus on inhibiting bone resorption but often have serious side effects. AF-MSCs and their derived EVs have demonstrated potential in addressing this gap (107). In an osteoporosis model, AF-MSC-EVs enhanced the differentiation and viability of human pre-osteoblasts, reduced apoptotic markers, and mitigated oxidative stress induced by dexamethasone. These findings suggest that AF-MSC-EVs can restore precursor cell function and slow steroid-related bone loss (15, 108).

Focal cartilage defects are a significant risk factor for developing osteoarthritis (OA) and are prevalent among both older adults and younger populations. Over the past decade, several animal studies have explored the use of AF-MSCs in repairing osteochondral defects (OCD). For instance, AF-MSC sheets in a rabbit knee OCD model demonstrated the ability to repair cartilage by surviving and differentiating into chondrocytes at the healing site, making them a promising tool in cartilage tissue engineering (109, 110). Moreover, injecting AFSC-EVs in a rat OA model resulted in superior pain tolerance, improved histological scores, and increased cartilage matrix synthesis compared to MSC-treated defects. This cell-free approach could overcome many limitations of traditional cell therapies (111, 112). Similarly, a cocktail of AF-MSCs and hyaluronic acid injected into a rat OA model showed notable cartilage regeneration, highlighting a strategy that is non-tumorigenic, ethically acceptable, and immunomodulatory (113).

Neurodegenerative diseases

The relationship between autoimmunity and neurodegeneration is complex, with evidence suggesting that immune processes may contribute to the progression of various neurodegenerative diseases. Autoimmune diseases arise from immune attacks on the body's own tissues, while neurodegenerative diseases primarily involve the death of neurons caused by factors such as protein aggregation, mitochondrial dysfunction, and oxidative stress. These factors can sometimes trigger secondary immune responses (114). Some neurodegenerative diseases, such as multiple sclerosis (MS), are clear autoimmune disorders in which the immune

system directly attacks the nervous system. In contrast, other conditions like Alzheimer's disease (AD), Parkinson's disease, and amyotrophic lateral sclerosis (ALS) show inflammatory or autoimmune-like features but are not classified as autoimmune diseases. In these instances, the immune system may play a secondary role, responding to existing neuronal damage rather than initiating it (115).

Current therapies for neurodegenerative diseases primarily alleviate symptoms without addressing the underlying pathogenesis. Recent studies highlight the roles of neuroinflammation, apoptosis, and oxidative stress in these diseases (116). MSCs, in particular hAF-MSCs, exhibit anti-apoptotic, anti-inflammatory, and antioxidative effects, making them promising candidates for treating neurodegenerative diseases. AF-MSCs have shown promise in rodent models for conditions such as hypoxic-ischemic encephalopathy, periventricular leukomalacia, and myelomeningocele, where they suppress neuronal inflammation and restore neuronal cells (117).

Zheng et al. studied hAM-MSC transplantation in APP/PS1 double-transgenic mice, a model of Alzheimer's disease (AD). The transplantation significantly reduced amyloid-β (Aβ) deposition and improved spatial learning and memory deficits. These benefits were linked to increased Aβ-degrading factors, activated microglia, and modulation of neuroinflammation. Additionally, enhanced hippocampal neurogenesis in the dentate gyrus and improved synaptic plasticity likely contributed to cognitive recovery in the mice. The improved cognition appears to result from increased hippocampal synaptic density and neurogenesis driven by brain-derived neurotrophic factor (BDNF) (118). AF-MSCs have demonstrated neurotrophic effects on Schwann cells. In animal models with nerve crush injuries, AF-MSCs improved electrophysiological function, nerve myelination, and the expression of neurotrophic factors (119). In a rat model of spinal cord injury, transplanted hAF-MSCs enhanced neurogenesis and suppressed astrogliosis at the lesion site, suggesting their potential for restorative therapy (120).

Exosomes derived from hAF-MSCs have a high potential for promoting cell survival and possessing antioxidant properties. They have been shown to reduce inflammation caused by microglia and mitigate neurotoxicity. Therefore, they could be effective in treating neurological conditions associated with inflammation, such as Alzheimer's disease (121). Additionally, hAF-MSC-Exo exhibit significant neuroprotective effects through anti-apoptotic and pro-survival pathways that are influenced by their miRNA content, as demonstrated in an in vitro ischemia-reperfusion model (122).

Despite promising results from preclinical studies, MSC-based therapies face several challenges, including immune rejection and inconsistent clinical outcomes. Variability among patient populations, different MSC sources, and various delivery methods contribute to these inconsistencies. Additionally, therapeutic interventions targeting the central nervous system (CNS) encounter further difficulties due to the protective barrier of the blood-brain barrier (BBB) (116). MSC-based delivery systems and exosomes have been explored as potential solutions to improve drug delivery across the

BBB. These systems have shown the capability to efficiently deliver drugs and penetrate brain tissues, offering an alternative to traditional monoclonal antibody (mAb) therapies (123, 124). Integrating MSC delivery systems with antibody therapies might enhance treatment efficacy, but this approach requires optimization to resolve issues such as lysosomal degradation and immune compatibility (116). Further research is essential to standardize protocols and evaluate the long-term safety and efficacy of these therapies.

Conclusion

AF and its derivatives, including EVs, exosomes, and AF-MSCs, offer unparalleled potential in regenerative medicine. The high concentration of EVs in AF and their potent immunomodulatory properties stemming from the origination of the fetoplacental unit positions them as promising therapeutic agents for treating inflammatory conditions and modulating immune responses. AF-EVs, and AF-MSCs and their secretome, i.e. AF-MSC-EVs and AF-MSC-Exo, have shown remarkable efficacy in a wide range of applications, including the regeneration of cardiac tissue, bone, cartilage, and internal organs, as well as in drug delivery and the treatment of neurodegenerative diseases. Innovations in ultracentrifugation techniques have further enhanced the clinical potential of EVs and exosomes by facilitating their delivery in smaller, more manageable volumes. Additionally, their capacity to encapsulate and protect RNA cargo within lipid membranes ensures stability and functionality, underscoring their versatility in therapeutic and diagnostic contexts. Emerging evidence underscores the transformative potential of AF and its derivatives in addressing complex medical challenges through minimally invasive and highly effective interventions. These advances establish a robust foundation for future clinical applications and propel the ongoing exploration of AF-based regenerative therapies.

Conflict of interest

The authors have no conflict of interest to declare.

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