Cell-Based Therapy Approaches in Treatment of Non-obstructive Azoospermia

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Abstract
The rate of infertility has globally increased in recent years for a variety of reasons. One of the main causes of infertility in men is azoospermia that is defined by the absence of sperm in the ejaculate and classified into two categories: obstructive azoospermia and non-obstructive azoospermia. In non-obstructive azoospermia, genital ducts are not obstructed, but the testicles do not produce sperm at all, due to various reasons. Non-obstructive azoospermia in most cases has no therapeutic options other than assisted reproductive techniques, which in most cases require sperm donors. Here we discuss cell-based therapy approaches to restore fertility in men with non-obstructive azoospermia including cell-based therapies of non-obstructive azoospermia using regenerative medicine and cell-based therapies of non-obstructive azoospermia by paracrine and anti-inflammatory pathway, technical and ethical challenges for using different cell sources and alternative options will be described, and then the more effectual approaches will be mentioned as future trends.

Keywords Non-obstructive azoospermia · Cell therapy · Regenerative medicine · Paracrine effect · Inflammation

Introduction
Male factors account for about 50% of couples’ causes of infertility. Among these, non-obstructive azoospermia (NOA) constitutes 10–15% of male infertility and 60% of azoospermic men, with an impaired spermatogenesis leading to a lack of sperm in ejaculation. NOA has been shown to occur as a result of congenital or genetic abnormalities, endocrine disorders, varicocele, trauma, exposure to gonadotoxins, infectious agents, chemotherapy drugs, and idiopathic causes [1].

The etiology of NOA is generally categorized into two origins: primary hypogonadism due to primary testicular failure and secondary (hypogonadotropic) hypogonadism due to hormonal abnormality [2].

In secondary hypogonadism, hormone replacement therapy can be administrated to stimulate spermatogenesis and restore fertility with or without the need for surgery [3].

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In primary hypogonadism of NOA the main focus of treatment is on sperm retrieval through testicular sperm extraction.

Therefore, as long as genetic abnormalities such as Klinefelter syndrome and Y chromosome microdeletion do not cause primary testicular failure and are not the main cause of infertility, NOA patients can benefit from hormonal or surgical treatments.

On the other hand, despite hormonal or surgical therapeutic methods, the outcomes of NOA treatments, especially for primary testicular failure, are usually unsatisfactory. In primary hypogonadism of NOA, if sperm could not be retrieved by testicular sperm extraction, the only current option relies on using donor sperm or adoption which deprives couples of having a related biological child.

The larger category of primary testicular failure of NOA is due to idiopathic or inflammatory causes for which there is no effective treatment option [4].

Nowadays, cell transplantation can be used to treat male infertility, especially for the treatment of NOA, in two ways: one from regenerative medicine view and for treatment resulting from failure of germ cells proliferation and differentiation and the other with the help of their paracrine and anti-inflammatory effects for treatment of NOA resulting from idiopathic and inflammatory problems.

Now we will discuss here about the cell-based therapies for NOA using therapeutic approaches of stem/stromal cells based on regenerative medicine and the paracrine mechanisms.

**Cell-Based Therapies of NOA Using Regenerative Medicine**

From a regenerative medicine view, there are two experimental approaches in which germ cell colonies can be produced for restoring fertility in men with NOA: (1) in vivo approach in which spermatogonial stem cells (SSCs), as the precursors to mature spermatids, are transplanted into the seminiferous tubules of infertile individual. (2) Based on in vitro studies, in addition to SSCs, embryonic stem cells (ESCs) [5], induced pluripotent stem cells (iPSCs) [6], and mesenchymal stem cells (MSCs) [7] can be cultured in vitro and differentiated into male germ cells.

In the following, restoring fertility in men with NOA using mentioned cell sources will be discussed (Fig. 1a).

**Spermatogonial Stem Cell Transplantation**

SSCs are able to self-renew, differentiate, and regenerate spermatogenesis. To regulate the process, close interactions between SSCs and Sertoli cells surrounding the SSCs, and the creation of a microenvironment called the stem cell niche is essential.

Previously, Lim et al. found SSCs in the testes of NOA patients and isolated and cultured them under exogenous feeder-free culture conditions. After long-term culture, SSCs could be differentiated to male germ cells with developmental potential [8].

Generally, strategies for using SSCs to restore spermatogenesis and fertility include (1) harvest and grafting of testicular tissue and (2) injection of isolated SSCs.

For successful testicular tissue grafting, based on previous studies, SSCs cryopreservation via slow freezing with dimethyl sulfoxide, grafting time after sexual maturity of patients, grafting location in the scrotum, and high levels of gonadotropins, luteinizing hormone (LH) and follicle stimulating hormone (FSH), are ideal requirements.

Although testicular tissue grafting is very optimal in that the natural niche of stem cells is retained, in some cases, such as cancer patients, it needs to be more optimized because of concerns about cancer cell contaminations [9–11].

For injection of isolated SSCs, among the injection targets, including seminiferous tubules, rete testis, and efferent ducts, rete testis injection with guidance of ultrasonography seems to be the most promising injection technique to date [12].

Following transplantation, SSCs migrate to the basement membrane of seminiferous tubules.

One of the advantages of this method over the testicular tissue grafting is that it can provide conception without the need for assisted reproductive techniques (ART) [13].

SSCs can be differentiated into germ cells through in vitro spermatogenesis; in this way, testicular tissues or isolated SSCs can be cultured for days and developed into spermatids. As previously, Lim et al. found SSCs in the testes of NOA patients and isolated and cultured them under exogenous feeder-free culture conditions. After long-term in vitro culture, SSCs could be differentiated to male germ cells with developmental potential [8].

However, despite promising research results of SSC transplantation-based therapy in animals specially in rodent models for restoring infertility, translating this approach to the clinic needs to be optimized in human models from different aspects including SSCs culture condition, cancer cells contamination in cancer patients, cryopreservation of the SSCs, ideal injection site for transplantation, safety of transplantation in recipients, frequency of injection, efficient SSCs volume, and dose for injection [12].

Thus more clinical researches are required to overcome these challenges for SSC transplantation-based therapy for NOA.

**Embryonic Stem Cells**

Due to the high differentiation potential of ESCs and the ability of these cells to produce germ cells with more efficiency and more autonomy, ESCs are more suitable options of cell therapy for spermatogenesis disorders in NOA.
Fig. 1 NOA cell-based therapy. a Approaches for cell-based therapies of NOA using regenerative medicine. b Cell-based therapies of NOA via anti-inflammatory and paracrine factors secreted by MSCs and DSCs.
Initially, in mice, it was shown that ES cells can differentiate into male germ cells via embryoid body (EB) formation combined with bone morphogenetic proteins (BMP4) or retinoic acid (RA) induction. After transplantation into mouse testes, ES-derived cells during spermatogenesis generate sperm [14].

Next studies also demonstrated the differentiation potential of ESCs to male germ cells, and that EB microenvironment supports male germ cell development and capacity of fertilizing oocytes [7, 15–17].

Thus, using ES cells to produce male primordial germ cells has promising applications for male infertility treatment in NOA. However, regarding the use of ESCs in infertility treatment, sperms derived from ES cells would be genetically unrelated to the patient. Moreover, limited sources of human ES cells, ethical challenges, and governmental concerns are the main barriers for their clinical applications [18].

**Induced Pluripotent Stem Cells**

Another source of pluripotent cells in addition to ESCs and SSCs is induced pluripotent stem cells (iPSCs). In comparison with ESCs, iPSCs are free of ethical concerns;
moreover, they have provided powerful means for personalized cell therapies.

Generally, producing male germ cells in vitro from pluripotent stem cells like ESCs, SSCs, and iPSCs can be done via two methods: embryoid body (EB) formation and the monolayer differentiation, in the presence of cytokine and growth factors [19].

Thus, patient-specific iPSCs can be established from NOA patients and recruited for producing male gametes through in vitro culture and transferring into testis tissue to restore spermatogenesis in idiopathic NOA without genetic abnormalities. But if NOA cases are caused by genetic disorders such as Klinefelter syndrome and Y chromosome microdeletion, this platform should be used to produce NOA-specific gametes for modeling and evaluating male infertility rather than for NOA cell therapy because iPSCs generated from NOA patients with genetic disorder showed compromised germ cell development potential [20].

**Mesenchymal Stem Cells**

Among stem cells with ability to differentiate to or induce proliferation of germ cells like SSCs, ESCs, and iPSCs, different sources of MSC have been used for this purpose. MSC therapy has the potential for direct application in vivo without limitations including immunogenicity, ethical concerns, and source scarcity (as in ESCs) or risk of forming teratomas and oncological and genetic instabilities (as in iPSCs and SSCs) or poor content in the source and isolation and culturing difficulties (as in SSCs) [21–23]. Different sources of MSCs especially MSCs from bone marrow, adipose tissue, and umbilical cord have been utilized for treatment of azoospermia in preclinical and clinical studies (Table 1) [24–30].

The potential of MSC therapy in treatment of male infertility can be exerted in the following mechanisms: (1) differentiation into the spermatozoa or merging with the endogenous SSCs to recover the spermatogenesis and (2) restoration of spermatogenesis via immunomodulatory and paracrine effect through secretion of growth factors and cytokines [31, 32].

As mentioned above, using MSCs for treatment of NOA through differentiation has been performed by different research groups. To date, many studies showed that transplanted bone marrow-derived MSCs (BM-MSCs) into the testis of busulfan-treated infertile animal models could differentiate into male germ cell and also Sertoli and Leydig cells [29, 30, 33, 34]. BM-MSCs are very similar to Sertoli cells and just like them are immune tolerant cells with the same embryonic origin. Transplanted BM-MSCs could reconstitute tubular microenvironment and provide the proliferation of inactivated germinal cells in the host tubules [35].

Although in an in vivo study, in an autoimmune infertility mice model, transplanting allogeneic BM-MSCs showed immunomodulatory effects on antibody production, but that was not a long-lasting immunomodulatory effect [56, 57].

Compared to cells from other sources, BM-MSCs have a high ability to proliferate and differentiate, and like all MSCs, have immunomodulating properties, but their differentiation ability and regenerative effects are greater than their anti-inflammatory and paracrine effects. Therefore, they are more suitable for regenerative purposes, but MSCs originating from fetal and perinatal tissues such as placenta-derived MSCs (PD-MSCs), amniotic membrane (AM-MSCs), amniotic fluid (AF-MSCs), fetal membrane (FM-MSC), and umbilical cord blood-derived MSCs (UC-MSCs) have higher properties of immune modulation and are more suitable for the paracrine pathway, especially for the purpose of immune modulation [58, 59].

MSC therapy of NOA via immunomodulatory mechanism has been successful in different animal studies, [55], and is discussed in the next section.

**Cell-Based Therapies of NOA by Paracrine and Anti-inflammatory Pathway**

It has been shown that immunological factors and inflammatory processes may be responsible for testicular damage and male infertility in about 30% of asymptomatic infertile patients [60, 61]. Studies have shown that infiltration of immune cells has been observed in at least 20% of testicular biopsies of infertile patients with azoospermia, which means inflammatory infertility has a significant contribution to male infertility [62, 63].

Previous studies also showed the presence of immune cell infiltration and corresponding inflammatory conditions in testicular biopsies of all dogs with NOA including M1 pro-inflammatory phenotype macrophages and pro-inflammatory monocytes and cytokines [64].

In previous studies, biopsies from NOA-affected men showed inflammatory lesions (including lymphocytes and monocytes/macrophages) associated with impaired spermatogenesis, while specimen from patients with OA indicated intact spermatogenesis without inflammation [65, 66]. That was also indicated that inflammatory effects can result in damage to the testicles and epididymis and that the levels of inflammation mediators such as tumor necrosis factor (TNF) and Activin A were elevated in human testicular biopsies with impaired spermatogenesis [67]. NOA has been also observed in 10% of men with acute epididymitis [68].

As mentioned in previous section, MSCs have been shown to have immunomodulatory, anti-inflammatory, anti-apoptotic, and proliferative effects through secretion of cytokines and growth factors.

MSC-derived exosomes, as part of their paracrine factors, also have similar functions to MSCs but with the superior
properties that is mentioned in the relevant section. In addition to MSCs and their exosomes, stromal cells isolated from decidua, known as decidua stromal cells (DSCs), have similar properties to MSCs with more potent immunomodulatory and anti-inflammatory effects.

In the following sections, the potentials for NOA cell therapy using MSCs, MSC-derived exosomes, and DSCs through their immunomodulatory and anti-inflammatory effects are described (Fig. 1b).

**Mesenchymal Stem Cells**

Among different source of MSCs including bone marrow, adipose tissue, umbilical cord, endometrium, dental pulp, and menstrual blood, some of them have superior characteristics to others, making them in higher priority for cell therapy. For instance, endometrium and placenta-derived MSCs and adipose tissue-derived MSCs (AT-MSCs) have superior properties compared to bone marrow (BM)-MSCs, including greater immunomodulatory effects, higher secretion of cytokines and growth factors, and higher proliferation rate. However, many studies that have used MSCs for azoospermia have not confirmed that MSCs differentiate into spermatozoa or only through paracrine effects can induce reconstitution of the testis and epididymis tubes and recovery of spermatogenesis [28, 29, 42, 43, 48, 52, 69].

On the other hand, other studies have shown that MSCs are able both to differentiate into germ cells, in vitro and in vivo, and to improve the testicular tissue via paracrine effects [38, 47, 51, 52, 54].

Some studies have also shown that BM-MSCs were not capable of differentiation into sperm [70]. However, it is not yet clear whether transplanted stem cells differentiate into spermatocytes, but it can be concluded that if different sources of MSCs are not able to differentiate into sperm, they may improve testicular tissues and recover spermatogenesis through their paracrine secretions [55, 71].

In proportion to this purpose, using some MSCs sources such as placenta-derived MSCs due to their better immunomodulatory effects are more compatible for using in NOA.
cell therapy through paracrine and immunomodulatory pathway. Different mechanisms of action by which MSCs can induce spermatogenesis in the inflammatory environment have been summarized in Fig. 2.

There are promising and valuable results from preclinical researches and clinical trials using placenta-derived MSC (PD-MSCs) for treatment of infertility-related disorders. Placenta-derived MSC as a non-surgical treatment in men with Peyronie’s disease [72] and erectile dysfunction [73] were evaluated and resulted in outstanding results.

Therefore, it is better to utilize the appropriate MSC source purposefully, depending on the etiology of NOA and which pathway of treatment is to be used.

Preclinical studies showed that autologous MSCs could be transplanted into the testis and migrate and settle down in the seminiferous tubules of the basement membrane. Then, they can proliferate and differentiate into spermatogonia in some seminiferous tubules of the animal model. Also they could ameliorate testicular damage through paracrine effects such as anti-inflammatory, antioxidative, and anti-apoptotic factors [33, 41, 42].

Up to now, not many clinical trials have been recorded for cell therapy of NOA (Registered trials: NCT02414295, NCT02025270, NCT02008799, NCT02041910, NCT02641769, NCT03762967, RCT20190519043634N1), and none of the registered trials have been fully published to treat NOA yet.

Regarding cell-based therapies of NOA using regenerative and differentiation approach, the autologous source of MSCs is preferred so that the male germ cell produced by MSCs are genetically related. When there is a genetic disease in the parent, MSC therapy of NOA via regenerative and differentiation approach may be preferable using allogeneic source of MSC. In this regard, the only challenge is producing genetically unrelated male germ cells, which in many cases is not accepted by couples. On the other hand, along with aging, differentiation potential, viability, and the reservoir of MSCs decrease. Therefore, in NOA cell-based therapies by the paracrine and anti-inflammatory pathway, other options such as exosomes and DSC (low differentiation potential compared to MSC) are suggested as suitable alternatives for this purpose [74].

MSC-Derived Exosomes

MSC-derived exosomes, as part of extracellular vesicles (EVs), have similar properties and functions to MSCs but have very low immunogenicity and tumorigenicity compared to MSCs, with no differentiation potential, and they are well tolerated, easier, and more practical to use in vivo. Conditioned media (CM) of MSC culture contains the EVs with the same properties of MSCs [75].

In Zhankina et al. study, the effect of EV-contained CM in comparison with MSCs was studied for the first time to treat non-obstructive azoospermia in the NOA mice models. The results showed successful recovery of spermatogenesis in all therapy groups with more favorable results in MSCs compared with the CM group [55].

![Fig. 2](https://example.com/fig2.png)

**Fig. 2** Different mechanisms of action for MSCs in the inflammatory environment to retrieve spermatogenesis. IDO, indoleamine 2,3-dioxygenase; GCS, germ cell-specific
Other study showed that exosomes isolated from urine-derived stem cells could facilitate the recovery of spermatogenesis in busulfan-induced NOA mice [76]. Exosomes can exert their paracrine effects through carriage of lipids, proteins, miRNAs, and mRNAs into target cells [77, 78]. They have also proregenerative effects in damaged regions directly, just like stem cells [79]. In addition, they can regulate the function of target cell through regulation of target protein/gene expression [80].

As demonstrated in Guo et al. study, bone marrow MSC exosomes could restore spermatogenesis in NOA mice model through inhibiting the p38MAPK/ERK and AKT signaling pathways [81].

In other studies, exosomes derived from other cell types could improve spermatogenesis through above-mentioned mechanisms. In Mobarak et al. study, amniotic fluid-derived exosomes could ameliorate sperm quality and spermatogenesis in NOA rat models. Using exosomes resulted in significantly increased OCT-3/4 + cells in NOA rats [82]. Sertoli-derived exosomes could also improve spermatogenesis through the regulation of oxidative stress in NOA mice models.

Based on different preclinical studies, using paracrine MSCs-derived exosomes for NOA clinical studies seems to be promising [55, 76, 83].

### Decidua Stromal Cells

During the start of pregnancy for pregnancy, significant changes in endometrium stromal cells occur following decidualization process. The transformed stromal cells are called decidual stromal cells that are specialized morphologically and functionally. DSCs play role in identification, selection, and acceptance of allogeneic embryos and the in development of immune tolerance and protection of semi-allogeneic fetus [84].

DSCs have similar properties to MSCs, but the ability of DSCs in preventing alloreactivity is significantly better than other sources of stromal cells, and they have stronger immunomodulatory effect in comprise to other sources of MSCs [85, 86].

The priorities of DSCs over other sources of MSCs include smaller size, higher proliferation rate, higher resistance to oxidative conditions, higher expression of homing markers in order to achieve inflammatory target areas, higher ability to suppress immunity, much lower differentiation potential (highly compatible for using in cell therapy of NOA through paracrine and anti-inflammatory pathway), no tumorigenesis report, more therapeutic effect, higher survival rate after freezing, easier access, and the need for cell-to-cell contact to induce immunomodulatory effects [87–91].

DSCs have been shown to have even higher proliferative capacity and greater immunomodulatory properties than stromal cells from neonatal tissues such as the amnion and chorion [86, 92].

Relying on the above characteristics, DSCs seem to be better therapeutic candidates for cell therapy of NOA through paracrine and immunomodulatory approach than regeneration pathway compared to MSC and other stromal cells.

Therefore, DSCs may have higher potential especially for treatment of inflammation-related NOA.

Heretofore, DSCs have been used in clinical trials to treat graft-versus-host disease (GVHD) and hemorrhagic cystitis [93–95] and COVID-19-induced acute respiratory distress syndrome (ARDS), and in preclinical settings to treat recurrent spontaneous abortion, and have yielded promising results in both settings [96].

It also seems that DSCs have a higher potential for fighting inflammation in inflammatory environments, as their location and activity is in such an environment with higher oxidative stress and inflammatory mediators. Therefore, stromal cells from placenta are more suitable candidates for the treatment of various inflammatory disorders [97].

To date, mesenchymal-like cells isolated from different parts of human placenta including amnion, chorion, and decidua have been used in preclinical and clinical studies to treat various diseases [98, 99]. Among these, placenta-derived MSCs (PD-MSCs) have been used for treatment of infertility-related diseases such as premature ovarian failure (POF) [100–103], testicular failure [104], and male sexual problems such as Peyronie’s disease [72] and erectile dysfunction (ED) [73] and have promising results.

MSCs seem to be preferred for use in the regenerative pathway due to their superior differentiation properties over DSCs, but DSCs are more potent in their immunomodulatory properties and are better options for the treatment of idiopathic NOA associated with inflammation.

As mentioned in previous section, MSC-derived exosomes have very low immunogenicity and tumorigenicity compared to MSCs, with no differentiation potential and other superiorities. However, exosomes still have their own challenges like culture separation, cell phenotype, and quantification in clinical applications.

Thus, DSCs may be preferred candidates for cell-based therapies of NOA by paracrine and anti-inflammatory pathway, and using this strategy for the NOA treatment in clinical settings is strongly supported.

Scientists stand there at the beginning of the therapeutic path using DSCs for inflammatory disorders, and there is a need for more preliminary, preclinical, and clinical studies for this purpose. However, given that the outstanding preclinical and clinical results following the use of DSCs for the treatment of GVHD, and hemorrhagic cystitis following HSCT, and ARDS caused by COVID-19, as well as the results from preclinical studies in animal models of male and female infertility, and on the other
hand, with the superiority of DSCs over MSCs, we can hope to have a higher potential of DSCs to treat infertility-related disorders that are associated with inflammation like idiopathic and inflammation-related NOA.

It is noteworthy that, for the sake of safety, the frequency of injections, the injected cell dose, and the post-injection anticoagulation therapy such as heparin infusion should be based on the previous clinical trials similar to those of GVHD and COVID-19 ARDS.

Prospects for Future Direction

Currently, the use of new diagnostic and therapeutic technologies like genomics, proteomics, and artificial intelligence, along with conventional therapeutic techniques of surgery and hormone therapy, has been promising in the treatment of NOA. However, in many cases, this severe form of male infertility requires other promising treatments. Cell-based therapies of NOA, depending on the etiology, which is structural defects or idiopathic (one of the main causes of which is related to inflammatory factors), can be potentially used through differentiation of stem cells (SSCs, ESCs, iPSCs, and MSCs) or immunomodulatory effects (MSCs, their exosomes, and DSCs), respectively.

Among these, based on the results of in vitro, animal model studies, and a clinical trial, MSCs are in the top priority of regenerative medicine for treatment of NOA due to their high differentiation capacity, high proliferative potential, and similarity to embryonic stem cells of the testes [105].

These fibroblast-like MSCs have also paracrine actions and are able to secrete growth factors and signaling molecules to restore spermatogenesis especially through anti-inflammatory pathway. However, according to in vitro data, preclinical experiences, and recent clinical trials for treatment of inflammation-related disorders such as GVHD and ARDS, DSCs have stronger immunomodulatory properties and some other priorities over MSCs such as higher proliferation rate, higher resistance to oxidative conditions, smaller size, higher expression of homing markers in order to achieve inflammatory target areas, lower differentiation potential, higher survival rate after freezing, easier access, and safer and more reliable on the target site due to the need for cell-to-cell contact to induce immunomodulatory effects [87, 90, 106–108].

As mentioned, regarding MSC-derived exosomes, they have anti-inflammatory and paracrine effects just like the parents, but they still have their own limitations such as culture isolation, cell phenotype, and quantification in clinical applications [109].

After all, DSCs may be preferred candidates for the treatment of inflammation-related NOA, and using this approach in clinical trials for the treatment of NOA is strongly supported. Regarding to the safety issue, the injected DSCs doses, injection frequencies, and anticoagulant therapy after injection must be optimized based on previous clinical trials using DSCs in other inflammatory-related disorders like GVHD.

Conclusion

Current therapies for patients with non-obstructive azoospermia, if not treated with surgery and hormone therapy, are limited and need to retrieve normal and mature spermatozoa. By cell-based therapies of NOA using two approaches based on regenerative medicine and the paracrine mechanisms, male germ cell could be produced from different cell sources such as SSCs, iPSCs, ESCs, and MSCs in vitro/in vivo (regenerative medicine), or spermatogenesis could be recovered using paracrine effects of secreting stromal cells/stromal cell derivatives such as MSCs, MSC-derived exosomes, and DSCs. In the regenerative pathway, each source of cells that male germ cell differentiate from has their own challenges.

In the NOA cell-based therapies by regenerative pathway, choosing the right MSC source may not be easy since some sources are not capable to differentiate to sperm, but in the paracrine pathway, MSCs that have higher immunomodulatory effects, such as placenta-derived MSC and endometrium MSC, appear to be appropriate sources for this purpose. DSCs can be a preferred candidate for NOA cell-based therapies using paracrine and anti-inflammatory approach due to superior priorities of DSCs over other sources of MSCs and their higher immunomodulatory effects. However, there should be more comparing studies between the different cell sources for treatment of NOA in vitro and in vivo and clinical trials to translate preclinical results to the clinic. On the other hand, challenges regarding methods for cell isolation, culture, and complications of achieving an appropriate and safe cell source should be somewhat resolved in order to take firm and serious steps in cell therapy of NOA.

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1. contributions to the conception of the work; data gathering and interpretation of data for the work
2. drafting the work or revising it critically for important intellectual content
3. final approval of the version to be published
4. agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved

We confirm that the manuscript has been read and approved by all named authors.
We confirm that the order of authors listed in the manuscript has been approved by all named authors.

**Declarations**

**Conflict of Interest** The authors declare no competing interests.

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