Cell Journal (Yakhteh)

Guide for Authors

Aims and Scope: The "Cell Journal (Yakhteh)" is a peer review and monthly English publication of Royan Institute of Iran. The aim of the journal is to disseminate information by publishing the most recent scientific research studies based on medical and developmental biology including cell therapy and regenerative medicine, stem cell biology reproductive medicine, medical genetics, immunology, oncology, clinical biochemistry, neuroscience, and tissue engineering. **Cell J**, has been certified by the Ministry of Culture and Islamic Guidance since 1999 and accredited as a scientific and research journal by HBI (Health and Biomedical Information) Journal Accreditation Commission since 2000 which is an open access journal. **This journal holds the membership of the Committee on Publication Ethics (COPE).**

1. Types of articles

The articles in the field of Cellular and Molecular can be considered for publications in **Cell J.** These articles are as below:

A. Original articles

Original articles are scientific reports of the original research studies. The article consists of English Abstract (structured), Introduction, Materials and Methods, Results, Discussion, Conclusion, Acknowledgements, Author's Contributions, and References **(Up to 40).**

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IN THE NAME OF GOD

Dr. Saeed Kazemi Ashtiani

Gone But not Forgotten

In the memory of the late Director of Royan Institute, Founder of Stem Cells Research in Iran and Chairman of ***Cell Journal*** *(Yakhteh)*. May he rest in peace.

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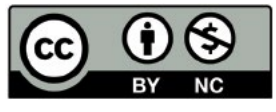

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Review Article

Original Articles

Letter to The Editor

Correction

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Mesenchymal Stem Cells in Regenerative Medicine, Possible Applications in The Restoration of Spermatogenesis: A Review

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Abstract

Infertility is a common clinical condition and about half of the major causes are due to male-related infertility. Pathogenesis of this abnormality is generally undefined; so establishing a proper treatment option is relatively uncertain. In recent years, several evidences demonstrated that mesenchymal stem cells (MSCs) can be a hope for innovative and efficient treatment of male infertility. This study reviews possible applications of MSCs in the restoration of spermatogenesis in male infertility of both humans and animals to suggest new avenues for future clinical practices. Articles published in "PubMed" and "Google Scholar" from January 1, 2000, to August 1, 2023, were investigated by searching items of "mesenchymal stem cells", "cell therapy", "cell transplantation", and, "regenerative medicine" keywords, in addition to the "urology", "andrology", "reproductive medicine", "male infertility", "azoospermia", and "spermatogenesis". The results obtained from the transplantation of MSCs in the treatment of male infertility seemed encouraging and they revealed the safety and efficacy of these cells to recover spermatogenesis; eventhough further stem cell research is still required before recruiting clinical application of MSCs in the treatment of human male infertility. Undertaking more well-defined, standardized, and reproducible protocols and enrolling larger sample sizes during a longer follow-up period can benefit the relevance of MSC transplantation in the restoration of spermatogenesis and treatment of male infertility. It seems that developing and utilizing stem cell transplantations, exosomes, scaffold delivery systems, and three dimensional (3D) culture methods may open a new window to getting more benefits from cell therapy in the treatment of men infertility.

Keywords: Azoospermia, Male Infertility, Mesenchymal Stem Cell Transplantation, Reproductive Medicine, Spermatogenesis

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Introduction

Infertility is still a common clinical problem influencing both genders, while approximately half of the infertility is male-related. Various terms have been used about male infertility to define sperm abnormalities, including aspermia, oligospermia or oligozoospermia, azoospermia, asthenospermia or asthenozoospermia, teratospermia or teratozoospermia, pyospermia or leukocytospermia and necrospermia (1). Aspermia is described by the complete absence of semen. Oligospermia is defined as the presence of few number of sperms in a semen specimen. Azoospermia is a condition with no sperm in semen. This condition is different from aspermia which has no semen. Asthenospermia is determined as a reduction in the percentage of motile sperm in the sperm specimen. Teratospermia is known as the presence of a large number of spermatozoa with abnormal morphology. Pyospermia is defined as a situation with the abnormal presence of white blood cells and infection in the reproductive tract and semen. Finally, necrospermia is known as a state whereby spermatozoa in the seminal fluid are dead and motionless (2).

Therapeutic strategies in male infertility have mostly focused on the improvement of sperm quality, hormone replacement therapy, assisted reproductive technology (ART), and surgery. Sperm cryopreservation has also been offered as a safe and effective way to preserve fertility in cancer patients before they undergo chemotherapy, radiation, or surgery. These conventional methods in the treatment of male infertility may be difficult to employ from a psychological and clinical point of view (2). To overcome these difficulties, regenerative medicine has opened a new door in the remedy of male infertility by utilizing cell transplantation. In cell transplantation, various stem cells including embryonic stem cells (ESCs), induced pluripotent stem cells (iPSCs), mesenchymal stem cells (MSCs), and spermatogonial stem cells (SSCs) have

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been used in the repair or regeneration of reproductive organs (3).

Among the stem cells, long term application of human SSCs is not easily possible (4). Applying iPSCs in the treatment of male infertility is limited due to the high risk of rejection, tumour formation and less potential of mimicking germ cell fate. ESCs are also limited since their current sources, obtained from human embryos and aborted fetuses, bear ethical issues in addition to having high risk of rejection and tumor formation (3). In this relation, MSCs have minimal ethical concerns, while they can be isolated easily from the various tissue resources, and differentiate or trans-differentiate into the multi-lineage cells. They can also secrete paracrine factors and microRNAs (miRNAs) to employ the resident stem cells to play an important role in tissue regeneration, or after cell transplantation to help fusion with the local cells (5). MSCs can create a supportive environment for SSCs to restore endogenous spermatogenesis. *The in vitro* differential potential of various MSC sources in germ cells has been previously described (6). Therefore, this review presented possible regenerative medicine applications of MSCs in the restoration of spermatogenesis.

Sources and selection criteria

Articles published in "PubMed" and "Google Scholar" from January 1st, 2000 to August 1st, 2023, were investigated by searching "mesenchymal stem cells", "cell therapy", "cell transplantation", and "regenerative medicine" terms, in addition to, "urology", "andrology", "reproductive medicine", "male infertility", "azoospermia", and "spermatogenesis".

Regenerative medicine

By utilizing stem cells, scaffolds, and biomaterials, regenerative medicine is considered a modern discipline which can recruit both basic and clinical sciences in the treatment of different diseases. Among different categories of stem cells, MSCs, based on their paracrine effects, have a prominent role in the regeneration of damaged tissues (5). MSC differentiation into germ lines looks a promising therapeutic approach in the treatment of male infertility (7) and in this regard, various MSC sources have successfully been differentiated *in vitro* into germ cells (6, 7).

Characteristics of mesenchymal stem cell

MSCs have the ability of self-renewal, easy expansion and differentiation into cells of different lineages which makes them a proper candidate for cell therapy (8). They are multipotent and they can be derived from various sources, such as bone marrow, adipose tissue, dental pulp and Wharton jelly (5, 8). They are positive for expression of mesenchymal cell surface markers such as CD9, CD13, CD29, CD44, CD47, CD49, CD71, CD54, CD56, CD59, CD73, CD81, CD90, CD98, CD105, CD106, CD120a, CD124, CD140, CD146, CD147, CD151, CD166, CD276 and negative for hematopoietic cell markers such as CD34 and CD45 (5, 8, 9).

MSCs with low immunogenicity can actively participate in the healing of damaged tissue. They secrete immunoregulatory factors that can inhibit the proliferation and function of Thelper 1 (Th1)/Th17 cells, the antigen-presenting function of dendritic cells and macrophages resulting in anti-apoptotic and anti-inflammatory activities. MSCs play anti-inflammatory roles by increasing the secretion of interleukin-4 (IL-4), IL-10, IL-11, IL-13, and transforming growth factor beta (TGF-β), in addition to decreasing the secretion of IL-6, IL-12, IL-21, IL-23 and reduction in the activity of nuclear factor kappa-light-chain-enhancer of activated B (NF-κB) cells (5, 8, 9). Their paracrine properties in the regulation of cell proliferation, survival, and differentiation play an important role in immunomodulation too. They also contribute to angiogenesis and tissue homeostasis which can promote tissue regeneration (5, 8). In a systemic review, it was shown that MSCs from various tissue sources could successfully be differentiated into germ cells or gamete progenitor cells. Successful allotransplantation of these cells or xenotransplantation as well as utilizing their exosomes can be effective in the treatment of non-obstructive azoospermia (NOA) of animal models to restore spermatogenesis and fertility (9). So scientists focused on MSC roles in rehabilitating the microenvironment of spermatogenesis and treatment of male infertility (Fig.1) (7).

Male infertility

Mammalian cells are divided into somatic and germ cells, based on their functions, while germ cells are involved in survival and reproduction. In males, the testis consists of germ cells and other cell types, such as Sertoli and Leydig cells for nourishing and supporting the development of germ cells and spermatogenesis (1). Spermatogenesis is an organized and dynamic cell differentiation started in the early fetus and it is completed after puberty by undergoing different stages of mitosis, meiosis, and finally spermatogenesis (6). For meiosis and the protection of haploid cells from the immune system in spermatogenesis, the blood-testis barrier (BTB) has a crucial role (1, 6).

Several factors can affect spermatogenesis. Advancing age can lead to germ cell apoptosis and abnormal spermatogenesis (1, 10). Since 85% of the testicular parenchyma participates in spermatogenesis, a reduction in the size of the testes can result in less sperm production (6). Additionally, a decrease in DNA integrity can damage the development of germ cells. It can also lead to the formation of abnormal motility, morphology, and function of the sperm, subsequently resulting in male infertility (11). Radiation and chemotherapy are well-known gonadotoxic factors that can negatively impact spermatogenesis, damage the somatic environment within the testis, impede spermatogonial differentiation and lead to male infertility (1, 6, 11).

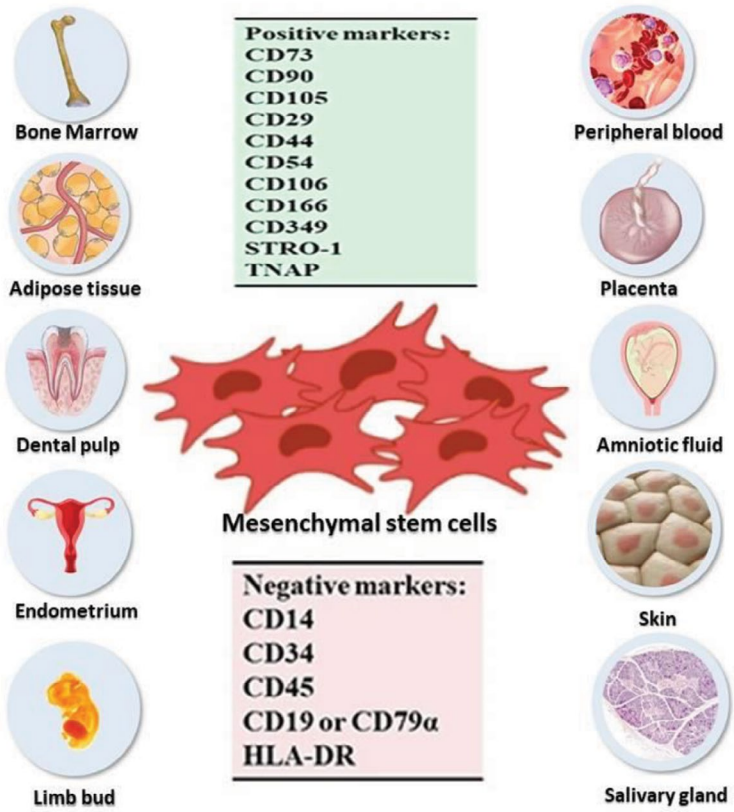

Fig.1: Sources and markers of mesenchymal stem cells.

Globally, 30 million men were reported to suffer from infertility (2). World Health Organization (WHO) report has defined a marriage as infertile when no pregnancy happens within 12 months of unprotected sex (12). Assessment of male infertility consists of a thorough history and physical examination to check abnormalities in the testes, vas deferens, or seminal vesicles, as well as investigation of risk factors for infertility, such as primary or congenital, and acquired or idiopathic causes (Fig.2) (6, 11).

Male infertility is categorized into different types. The absence of sperm in semen and ejaculation is defined as azoospermia. This abnormality is subclassified into two categories, including pretesticular and testicular (NOA), and obstructive azoospermia (OA, post-testicular) (1, 6, 13). In the OA, bilateral distal or proximal obstruction of the reproductive ducts happens, but the spermatogenic process is unaffected. In the NOA, primary or secondary testicular failure occurs and a phenotypic manifestation of three different types of testis histology can be seen, including the sertoli-cell-only syndrome (SCOS), maturation arrest (MA) at different stages of germ cell maturation like, spermatogonial arrest (SGA) and spermatocyte arrest (SCA)- and finally hypospermatogenesis. In azoospermia, almost 40% of the cases are defined as OA and around 60% are determined as NOA. To verify azoospermia, a repeated semen analysis and a hormonal assay would be beneficial. In the NOA, pre-testicular and testicular causes were described (12). In the OA, post-testicular causes were demonstrated, the majority of which were related to obstructions of ducts in the male reproductive system (13).

The major conventional treatment methods for male infertility focus on improving sperm quality which is dependent on etiologic factors (9, 13). SSCs are the basis for spermatogenesis and fertility in males. Damaging these cells may irreversibly alter the spermatogenesis process and lead to NOA. The only conventional solutions to achieve biological fatherhood are assisted reproductive technologies (ARTs), such as testicular biopsy, or testicular sperm extraction (TESE) in combination with intracytoplasmic sperm injections (ICSI), adoption or sperm donation (12).

Transplantation of own cryostored SSCs has been mentioned as a promising technique for fertility restoration when the SSC pool has been depleted (12). However, SSC transplantation in the treatment of male infertility has also several obstacles and challenges, including the efficiency of cryopreservation, experience and knowledge regarding cryopreservation of SSCs, exclusion of malignant cell contamination in cancer patients, and socio-cultural attitudes (11). As TESE-ICSI has shown limited success in NOA and there are many shortcomings with the transplantation of SCCs, there is a need to overcome these challenges (12). Transplantation of MSCs via their self-renewal properties and their ability to differentiate into multiple lineages may be a treatment of choice in male infertility, especially in patients with NOA (1, 6, 9). So based on these properties, MSC transplantations in male infertility can promote spermatogenesis due to their paracrine activities. This can affect Leydig cells by secretion of testosterone for sperm production (6, 9).

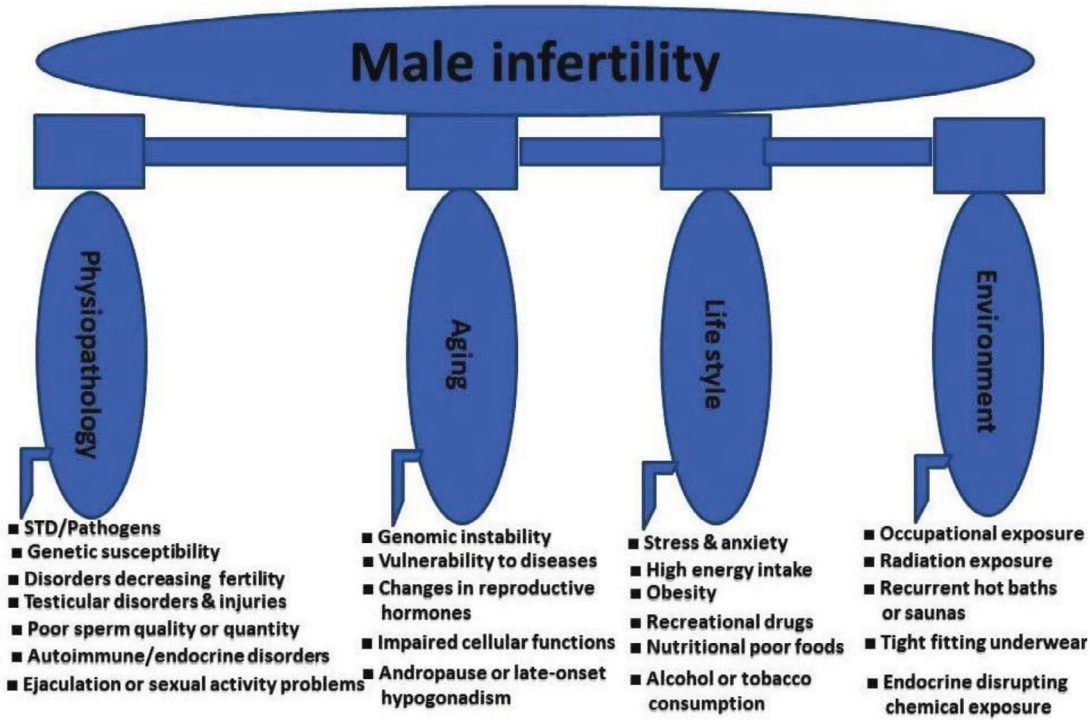

Fig.2: Multifactorial etiology of male infertility.

mesenchymal stem cell transplantation in male infertility

The final goal of fertility treatment is to provide pregnancy and to reach a successful outcome. In animal and human studies, it seems that regenerative medicine by applying cell transplantation, tissue engineering and growth factors has overcome the therapeutic drawbacks and it has opened a new window to improve the tissue or organ normal function and to enhance the fertilization capacity. Cell transplantation can utilize various stem cell sources in the treatment of infertility. In fertility treatment, animal and human studies have been divided into two major categories: i. *In vitro* differentiation into germ cells or gametes and ii. *In vivo* stem cell transplantation into reproductive organs. In the case of specifically transplanting into the testis, MSC can promote the local microenvironment of spermatogenesis to restore the spermatogenic process via secretion of nutritional factors after transplantation (14-21).

***In vitro* utilization of mesenchymal stem cells for the generation of male germ cells**

MSCs from different sources have been used *in vitro* for the generation of male germ cells, such as umbilical cord stem cells (UCSCs) (14-16), adipose tissue derived stem cells (AdSCs) (16-18), bone marrow derived stem cells (BMSCs) (17, 19), and Wharton's jelly stem cells (WJSCs) (20). Table 1 shows *in vitro* studies of MSCs sorted from different sources to differentiate into germ cells. By incubating human UCSCs with retinoic acid (RA), testosterone, and conditioned medium of testicular cell cultures showed high expression levels of male germ cell markers as well as proteins (14, 15). Human umbilical cord perivascular cells (UCPVCs) as a promising cell source, were utilized in transdifferentiation of germ cells and preservation of male fertility (16). For AdSCs, *in vitro*, transdifferentiating of human AdSCs into male germlike cells was demonstrated by rabbit Sertoli cells, while they expressed germ cell-specific markers (17, 21). ADSCs have been reprogrammed into primordial germ cell-like cells, and they had lower efficiency in comparison with AdSC-derived iPSCs (22). Comparing AdSCs with BMSCs, by adding BMP-4 to the culture medium, it was shown that the differentiation potential of BMSCs into primordial germ cells was better than AdSCs (23). AdSCs and BMSCs in the presence of RA were reported to have differentiation potential into male germ cells and expressed germ cell markers, like Mvh, Dazl, Stra8, and Scp3 (17). Kumar et al. (24) illustrated that adding RA to the culture medium of BMSCs could induce differentiation into germ cell-like cells. RA and titanium nanotubes coated with fibrin were demonstrated to offer a proper two-dimensional (2D) scaffold for BMSCs to differentiate into germ-like cells using *in vitro* maturation (IVM) and ART (25). Moreover, the efficacy of BMSCs on survival and expansion of the mice spermatogonial stem/progenitor cells (SSPC) and their differentiation to round spermatids was illustrated (26). BMSCs in the presence of testicular cells were shown to increase the expression of the male germ cell-specific genes (17, 19, 20). Ge et al. (27) showed that murine skin-derived multipotent papillary dermal fibroblast progenitors had *the* potential to generate male germ line cells. Table 1 presents *in vitro* studies of MSCs from different sources to differentiate into germ cells.

Table 1: *In vitro* studies of MSCs from different sources to transdifferentiate into germ-like cells

Type of stem cell	Other treatments	Outcome	Reference
UCSCs	RA, MPLA	Induction of migration and differentiation into germ-like progenitor cells, expression of male germ cell markers as well as proteins	(14)
UCSCs	RA, testosterone, conditioned medium of testicular cell cultures	High expression levels of C-KIT, DAZL, PIWIL2, and DDX4 in mRNA and protein levels, differentiation potential into germ-like progenitors	(15)
UCPVCs	NA	Being promising cell sources to be utilized in the preservation of male fertility strategies	(16)
AdSCs, BMSCs	RA	Remarkable expression of germ cells characteristic markers (Mvh, Dazl, Stra8, and Scp3)	(17)
AdSCs	LIF, GDNF, EGF, RA	Increase in expression of male germ cell markers (c-Kit and Mvh), differentiation into germ-like progenitors	(18)
BMSCs	RA, testicular cells	Increased expression of germ cell-specific markers (Dazl, Piwil2 and Stra8), differentiation potential into germ-like progenitors	(19)
WJSCs	RA and Sertoli cell-conditioned medium	Transdifferentiation into advanced stages of germ cells with an increase in expression of male germ cell-specific genes	(20)
AdSCs	Rabbit Sertoli cells	Transdifferentiating human AdSCs into male germ-like cells, expressing germ cell-specific markers	(21)
AdSCs	Reprogramming	Differentiation into primordial germ cell-like cells, lower efficiency in comparison with AdSC-derived iPSCs	(22)
AdSCs, BMSCs	BMP-4	Differentiation into primordial germ cells, BMCs were better capable of differentiation into primordial germ cells	(23)
BMSCs	Retinoic acid	Differentiation into germ-like cells	(24)
BMSCs	Retinoic acid, titanium nanotubes-coated fibrin formation, ultraviolet radiation	Offering a proper 2D scaffold for transdifferentiated germ-like cells, IVM for ART	(25)
BMSCs	SSPC	Differentiation into round spermatids	(26)
Skin-derived multipotent papillary dermal fibroblast cells	NA	Generating cells of the male germline progenitors	(27)

AdSCs; Adipose tissue-derived stem cells, ART; Assisted reproductive technology, BMSCs; Bone marrow-derived stem cells, BMP-4; Bone morphogenic protein-4, EGF; Epidermal growth factor, GDNF; Glial cell line-derived neurotrophic factor, IVM; *In vitro* maturation, LIF; Leukemia inhibitory factor, MPLA; Monophosphoryl lipid A, NA; Not available, RA; Retinoic acid, SSPC; Spermatogonial stem/progenitor cells, UCPVCs; Umbilical cord perivascular cells, UCSCs; Umbilical cord stem cells, and WJSCs; Wharton's jelly stem cells.

Potential mechanisms in the treatment of male infertility by mesenchymal stem cells

At least, three potential mechanisms were described by which MSCs may improve recovery of spermatogenesis, mostly in rodent models of azoospermia, including: i. Their direct differentiation potential into gametes, Sertoli or Leydig cells (9, 17, 25, 26), ii. Their paracrine secretions and mechanisms can protect and promote regeneration of the testicular niche by directly regulating different stem cells and progenitors, and iii. Indirect effects via immunomodulation of both paracrine and non-paracrine mechanisms make them a therapeutic potential to regulate the survival of testicular niche cells or SSCs (9, 17, 25, 26). Various factors including fibroblast growth factor-2 (FGF2), stem cell factor (SCF), leukemia inhibitory factor (LIF), epidermal growth factor (EGF), bone morphogenetic protein-4 (BMP4), BMP6, FGF5, serpine, angiopoeitin pro-inflammatory M1 and anti-inflammatory M2 cells are involved in regulation of testicular niche and angiogenesis (9, 17, 25). These mechanisms can favor a regenerative environment for the resumption of spermatogenesis by SSCs after testicular injury (9, 17, 25). The effect of treatment with MSCs may be due to the presence of secretomes, like vascular endothelial

growth factor (VEGF), insulin-like growth factor (IGF), miRNA, and having exosomes that can regulate their differentiation and regeneration characteristics in injured tissues (9, 17, 25).

In vivo studies of mesenchymal stem cells in the treatment of male infertility

In the treatment of infertility by MSCs, various animal models were utilized including mouse (28), rat (29), hamster (30), Guinea pig (31), etc. In addition, different methods were applied to induce male infertility, such as the utilization of genotoxic chemicals -like busulfan and physical- and surgical methods (28). Other genotoxic chemicals, except busulfan (30) have been mentioned in the literature, including cyclophosphamide (32), cisplatin (33), cadmium chloride ($CdCl_2$) (34), calcium chloride ($CaCl_2$) (35, 36), doxorubicin hydrochloride (DOX) (30), ethane dimethanesulfonate (EDS) (37), lead (LN) (38), etc. Physical and surgical methods, such as electron beam irradiation (38) and testicular torsion (39), have respectively been used for inducing male infertility. In treatment of male infertility, various MSC sources have been transplanted in animal models, such as BMSCs (38, 40), AdSCs (10, 28, 33, 35), amniotic fluid-derived stem cells (AFSCs) (41), placental mesenchymal stem cells (PSCs) (37), UCPVCs (42), urine-derived stem cells (USCs) (43) and amnion (44).

Among various methods to induce male infertility such as gonadotoxic chemicals, busulfan as a chemotherapeutic agent is conventionally applied in the treatment of leukemia. It has also been utilized for induction of male infertility since busulfan causes ROS-mediated apoptosis, reduction of SSCs and subsequent fertility loss (28). In busulfan-induced male infertility, transplantation of MSCs was demonstrated as a therapeutic approach to restore fertility (45). Cyclophosphamide is a chemotherapeutic agent which has also been efficiently used as a gonadotoxic chemical to induce male infertility at one dose of 150 mg/kg intraperitoneally followed by daily injections of 8 mg/kg for seven days (46). Cisplatin or cisplatinum, also called cis–diamminedichloroplatinum II, as an antineoplastic drug against cancers, has been efficiently utilized for induction of male infertility by injection of a single dose of 7 mg/kg intraperitoneally (34). $CdCl_2$, as a highly toxic gonadotoxic metal, has also been employed to induce male infertility with intraperitoneal injection of 0.4 mg/kg for five weeks or at a single dose of 2.5 mg/testis/100 g body weight dissolved in 0.1 ml of normal saline (37, 47). DOX from the family of anthracycline antibiotics and as an anticancer drug has been effectively used as a gonadotoxic chemical for induction of male infertility by intraperitonial injection of three doses of 3 mg/kg every two days (29). Endocrine-disrupting substances (EDS) are the other gonadotoxic chemicals that have been utilized for the induction of male infertility at a single dose of 50 mg/kg (37). LN is another gonadotoxic chemical inducing male infertility when it is injected intravenously at a single dose of 23.3 mg/kg (38).

Among the physical methods to induce male infertility, exposure to electron beam irradiation, at doses 6, 8, 10, or 12 Gray (Gy) from a linear accelerator to the scrotum, has been introduced in the literature. Hyperthermia by long-term exposure to a temperature of 43°C is also a physical method of male infertility induction (38). Considering surgical methods to induce male infertility, testicular torsion for more than 360° and longer than 24 hours (such as 720° testicular torsion for 3-4 hours) has been in favor of infertility (39). Table 2 just demonstrates *in vivo* studies of busulfan, as a gonadotoxic chemical, to induce male infertility in different animal models, while treatment with the various MSC sources has also been described. Table 3 illustrates genotoxic chemicals, except for busulfan, together with physical methods and surgical procedures in different animal models to induce male infertility, while treatment with different MSC sources has been mentioned too.

In vivo studies of busulfan-induced male infertility treated by mesenchymal stem cell transplantation

As presented in Table 2, AdSCs, AdSCs-conditioned medium (AdSC-CM), AFSCs, BMSCs-CM, PSCs, UCPVCs, USCs and USC-exos (exosomes) have been used in the treatment of male infertility induced by busulfan in different animal models i.e. Guinea pig, hamster, mouse and rat). Several studies have successfully used AdSCs in the treatment of busulfan-induced male infertility. They showed an increase in sperm count and motility with normal morphology (28, 31), a repairing effect in the injured epithelial tissue of seminiferous tubules (30, 45), an upregulation in the expression of male germ cell-related markers of Oct4, Stella, Ddx4, Dazl, PGP9.5, Stra8, and ITGα6 as well as proteins of TGFβ-SMAD2/3, JAK2-STAT3, and AKT (48), and an increase in the total diameter, cellular diameter, cellular area, cross-sectional area and spermatogenesis index of the seminiferous tubules (49). Transdifferentiation of AdSCs into spermatogenic cells and recovery of spermatogenesis were also visible (50). When AdSCs, BMSCs and BMSCs conditioned media (BMSC-CM) were compared in the treatment of male infertility of busulfan-induced azoospermia, AdSCS were less favourable than BMSCs to recover spermatogenesis (51).

Many researchers have efficiently utilized BMSCs in the treatment of busulfan-induced male infertility and reported BMSCs to survive and reside in recipient seminiferous tubules, while expressing SSC marlers like Vasa, Stella, SMAD1, Dazl, GCNF, HSP90α, integrinβ1, and c-kit (52). Transplantation of BMSCs increased the overall tubular fertility index (TFI) and decreased the expression of DNMT3A and H4K5ac in germ cells (34). They also elevated expression of the meiosis-associated genes of *Miwi*, *Stra8*, *CyclinA1*, *Pgk2* and *Scp3* and Sertoli cell barrier functional factors, i.e. ICAM-1 and N-cadherin (40). An enhanced testosterone and estradiol serum levels, upregulated expression of germ cell-specific genes miRNA-21, miRNA-34b, miRNA-34c, miRNA-122, miRNA-449a, miRNA-449b, and miRNA-449c, downregulation expression of miRNA-19b, miRNA-100, miRNA-141, miRNA-146a, miRNA-429, and let-7a. Restoration of the disrupted expression of Ccnd1, E2F1, Myc, PLCXD3, and ERα were also found, following

the transplantation of BMSCs (53). There was an increase in the cellular and total diameters, as well as cellular and cross-sectional areas in the basement membrane of seminiferous and epididymis tubules together with an increase in the survival, migration, homing and differentiation of these cells (54, 55). This resulted in the restoration of spermatogonial structures, such as spermatogonia, primary spermatocytes, spermatids and sperms in seminiferous tubules and the Sertoli cells expressing FSH receptor, after BMSCs transplantation, suggesting efficacy of these cells in the treatment of male infertility (10, 34, 40, 54-66).

Researchers have also used PSCs in treatment of busulfan-induced male infertility and reported restoration of the disrupted spermatogenesis, improvement in semen parameters, an increase in testosterone level and testis size based on the promoted autophagy, protection against oxidative stress, decrease in testicular oxidative damage, increase in the expression of proliferation genes (*PCNA* and *KI67*), and finally decrease in apoptotic genes (*γ-H2AX*, *BRCA1*, and *PARP1*) (42). AFSCs are the other choice among the MSCS which have been successfully utilized in the treatment of busulfan-induced male infertility. This caused the resumption of spermatogenesis, due to attenuation of degenerative and oxidative changes in the testis (41). HUCPVCs were another alternative, among MSCS, applied to treat busulfan-induced male infertility and recover spermatogenesis (67). Transplantation of USCs and their exosomes (USC-exos) were also illustrated to be beneficial in the treatment of busulfan-induced male infertility to restore spermatogenesis, based on the increase in expression levels of spermatogenic genes (*Pou5f1*, *Prm1*, *SYCP3*, and *DAZL*) and proteins, such as UCHL1 (43). USCs were shown to affect the proliferation of spermatogonia verified by expression of the germ cell markers, like octamer-binding transcription factor 4 (OCT4), α6 integrin, c-Kit and VASA (68). UCSCs among MSCs were the other group of stem cells utilized to treat busulfan-induced male infertility and to improve spermatogenesis through an increase in mRNA of the genes related to meiosis (like *Vasa*, *SCP3*, and *PgK2*) together with a decrease in FSH and LH hormonal levels (69). UCSCs can lead to a rise in germ cell-specific genes of miwi and synaptonemal complex protein (Scp3) too (70). SSCs in the presence of MSCs were another therapeutic alternative to restore spermatogenesis in busulfan-induced male infertility by increasing TFI, and recovery of the endogenous SSCs (71). By investigating the fertility protective effects of human amnion MSCs (AMSCs) against busulfan-induced testis toxicity in mouse, it was shown that hAMSC could restore spermatogenesis and positively impact testosterone, testicular tissue, cell proliferation, cell apoptosis, oxidative damage and defense, and expression of GCS and meiosis genes (44). Table 2 reveals the results of *in vivo* studies using the various MSC sources in different animal models to treat busulfan-induced male infertility.

In vivo studies of MSC transplantation in male infertility induced by different methods except busulfan

When BMSCs were utilized in the treatment of male infertility induced by cyclophosphamide, restoration of testicular function and enhanced spermatogenesis happened through reducing apoptosis and phosphorylated levels of ERK, AKT, and p38MAPK proteins (32); treatment with this cell type also led to an improvement in androgen hormonal profile (46). When cisplatin was used to induce testicular damage, administration of AdSCs (34) or BMSCs (72) improved spermatogenesis based on the improvement of testicular architecture, and the increase in testosterone level (33). These MSCs could affect malondialdehyde (MDA), tumor necrosis factor-alpha (TNF-α), and gene expressions of Bax, inducible nitric oxide synthase (iNOS), caspase-3, and p38-MAPK (72).

In male infertility induced by intraperitoneal injection of $CdCl_2$, transplantation of SSCs and BMSCs restored fertility, by increasing TFI, in addition to decreasing expression levels of DNMT3A and H4K5ac in germ cells (71, 73). Restoration of fertility following transplantation of SSCs and BMSCs happened based on apoptosis of mitochondrial 3, decrease in expression levels of the apoptosis-associated proteins (Bim, Bax, Cytochrome C, Caspase-3, active-Caspase-3 and AIF), and increased expression levels of Bcl-2 (47). In electron beam irradiated testes of infertility models, BMSCs when transplanted intratesticularly, could recover spermatogenesis and fertility (38). In the Dox-induced model of infertility, transplantation of BMSCs in the testes could restore spermatogenesis by decreasing testicular oxidative stress, and MDA levels, in addition to increasing the antioxidant capacity of BMSCs (74). In contrast, when testicular torsion was utilized to induce male infertility, transplantation of BMSCs into the testis could not cause sperm formation and expression of germ cell-specific markers (like Oct4, Vasa and c-Kit) (75). In a model of autoimmune infertility, BMSC transplantation was found to have immunosuppressive effects on antibody production for antisperm antibody (ASA) (76).

BMSCs were reported to counteract the deleterious effects of cyclophosphamide- (77), Dox- (29), LN- (37) and cdcl2-induced infertilities (78). These cells have a considerable restorative impact on sex hormones, reducing MDA and testicular oxidative stress, increasing antioxidant capacities, amelioration of superoxide dismutase (SOD), glutathione peroxidase and catalase levels, and finally decrease of DNA alteration and fragmentation which can lead to improved spermatogenesis (29, 37, 77, 78). So, BMSCs play an important role in recovering testicular function and reestablishment of spermatogenesis by differentiation into sperm and Leydig cells. It also leads to modulation of serum testosterone, since Leydig cells are responsible for testosterone production (78). Additionally, transplantation of AdSCs was found to counteract the negative impacts of $CaCl_2$ (35) and testicular torsion-induced male infertility (79). These changes happen through reversing the imbalance of glycolysis in sperm and testis, increasing the expression of phosphoglycerate kinase 2, glyceraldehyde-3-phosphate dehydrogenase-spermatogenic, activating Akt, glycogen synthase kinase 3 (GSK3), glycolysis cascades and ATP production, together with increasing serum testosterone secretion and balancing FSH level which ultimately results in improvement of sperm function, particularly in sperm motility and spermatogenesis (35, 39, 79).

Table 2: *In vivo* studies of the treatment of busulfan-induced male infertility using MSCs extracted from various sources and different animal models

Type of stem cell	Other treatments	Animal model	Outcome	Reference
AdSCs	AdSCs-CM	Mouse	Increased number of testis cells, sperm count and motility, and length density of seminiferous tubules, ameliorative effects in mouse testes	(28)
AdSCs	BMSCs, BMSCs-CM	Mouse	Normal morphology of seminiferous tubules, and successful recovery of spermatogenesis, while BMSCs were more favorable than the other choices in the therapy of azoospermia	(51)
AdSCs	TM4 cells, RA, testosterone	Rat	Formation of bigger and tightly packed male germ-like cells feature colonies, up-regulation of expression of male germ cell-related markers (Oct4, Stella, Ddx4, Dazl, PGP9.5, Stra8, and ITGα6) and protein expression levels TGFβ-SMAD2/3, JAK2-STAT3, and AKT pathways	(48)
AdSCs	NA	Guinea pig	Normal spermatogenesis, recovery of spermatogenesis, treatment of azoospermic infertility	(31)
AdSCs	NA	Hamster	Normal repair of epithelial tissue in seminiferous tubules, presence of spermatozoa in epididymis tubes, treatment of azoospermia	(30)
AdSCs	NA	Mouse	Increase in total diameter, cellular diameter, cellular area, cross-sectional area and spermatogenesis index of the seminiferous tubules, restoration of spermatogenesis, treatment of azoospermia	(49)
AdSCs	NA	Rat	Normal morphology of seminiferous tubules, active spermatogenesis, treatment of azoospermia	(45)
AdSCs being GFP^+	NA	Rat	Recovery of spermatogenesis, transdifferentiation into spermatogenetic cells, being $GFP^+/VASA^+$ and $GFP^+/SCP1^+$, treatment of azoospermia	(50)
UCSCs	NA	Mouse	An increase in mRNA levels of genes related to meiosis (*Vasa*, *SCP3*, and *PgK2*), a decrease in FSH and LH levels, restoration of the tubules to normal architecture, improved testicular failure and spermatogenesis	(69)
UCSCs	NA	Mouse	Presence of a round cell shape differentiated spermatids and spermatozoa, migration to the basement of the tubule, expressing germ cell markers octamer-binding transcription factor 4, α6 integrin, C-kit, VASA, improvement in histological features, treatment of azoospermia	(68)
UCSCs	NA	Mouse	Increased levels of spermatogenic gene expression and protein of germ cell-specific genes (miwi, VASA, synaptonemal complex protein Scp3), recovery of spermatogenesis, treatment of azoospermia	(70)
UCPVC	NA	Mouse	Prevention of gonadotoxic drug-induced infertility, recovery of spermatogenesis	(67)
BMSCs	NA	Rat	Enhanced testosterone and estradiol serum levels, up-regulated expression of germ cell-specific genes (miRNA-21, miRNA-34b, miRNA-34c, miRNA-122, miRNA-449a, miRNA-449b, miRNA-449c), down-regulation of expression of miRNA-19b, miRNA-100, miRNA-141, miRNA-146a, miRNA-429, and let-7a, restoration of the disrupted expression of Ccnd1, E2F1, Myc, PLCXD3, ERα and AKT1, treatment of azoospermia	(53)
BMSCs	SSC transplantation by co-transplanting TGFβ1-MSCs	Mouse	Increase in overall TFI and litter sizes, decrease in expression of DNMT3A and H4K5ac in germ cells, restoration of fertility	(54)
BMSCs	NA	Mouse	An increase in cellular and total diameters, cellular and cross-sectional areas, spermatogenesis index and recovered spermatogenesis, treatment of azoospermia	(55)

Table 2: Continue

Type of stem cell	Other treatments	Animal model	Outcome	Reference
BMSCs	SSCs, TGFß1	Mouse	Spermatogenesis, significantly better tubular fertility index TFI, recovery of endogenous SSCs, increase in homing efficiency of the transplanted SSCs, treating infertility	(71)
BMSCs	NA	Hamster	Formation of spermatogonia, primary spermatocytes, spermatids and sperms in seminiferous tubules, treatment of azoospermia	(56)
BMSCs	NA	Guinea pig	Normal appearance of spermatogenesis, restoration of fertility, treatment of azoospermia	(57)
BMSCs	NA	Rat	Survival, migration, homing and differentiation of stem cells at the seminiferous tubules basement membrane, expression of spermatogonia markers (Dazl and Stella), treatment of azoospermia	(58)
BMSCs	NA	Rat	Normal morphology in seminiferous tubules, presence of spermatogenesis, treatment of azoospermic infertility	(10)
BMSCs	NA	Rat	Expression of CD106 and germ cell surface marker (c-kit), transdifferentiation into germ cells, repair of damaged seminiferous tubules, treatment of azoospermia	(59)
BMSCs	NA	Hamster	Normal morphology of epithelial tissue of seminiferous tubules, presence of spermatozoa in epididymis tubes, spermatogenesis in seminiferous tubules, treatment of azoospermic infertility	(60)
BMSCs	Conditioned media derived from cultured testicular Sertoli cells	Rat	Survival and homing at the basement membranes of seminiferous tubules, expression of molecular markers of spermatogonial stem cells and spermatogonia (Vasa, Stella, SMAD1, Dazl, GCNF, HSP90α, integrinβ1, and c-kit), absence of any tumor mass, immune response, or inflammatory reaction, enhancing endogenous fertility, treatment of azoospermia	(52)
BMSCs	NA	Rat	Increased testicular size, recovering androgen hormonal profile levels, resuming spermatogenesis, trans-differentiation into germ cells, restoration of testicular functions, treatment of azoospermia	(61)
BMSCs	NA	Rat	Restoration of spermatogenesis, differentiation to spermatogonia and spermatozoa in seminiferous tubules, and interstitial cells between tubules, treatment of azoospermia	(62)
BMSCs	NA	Mouse	Differentiation into male germ cells, spermatogenesis, treatment of azoospermia	(63)
BMSCs	NA	Rat	Detection of MSCs in the seminiferous tubules, gene expression of a primordial germ cell marker (VASA), stem cell-specific markers (Oct4, SSEA-1 and SSEA-3), specific molecular markers of germ cells (c-Kit, Daz1; premeiotic marker Daz1 and post-meiotic markers c- Kit, Stra 8), presence of spermatocytes and spermatids in testicular tissue, transdifferentiation into germ cells, treatment of azoospermia	(64)
BMSCs- conditioned media (CM)	NA	Mouse	MSC-CM with the most spermatogenic colonies, but no spermatids; higher expressions of the meiosis-associated genes (*Dazl*, *Vasa*, *Miwi*, *Stra8*, *CyclinA1*, *Pgk2* and *Scp3*) in MSC-CM testis; increased levels of Sertoli cell barrier functional factors (ICAM-1 and N-cadherin); significantly improved the short-term restoration of spermatogonial structures	(40)
BMSCs (RFP transfected), SSCs	TGFβ1	Mouse	TFI after SSCT was similar to that after MSC-SSCT, donor-derived TFI after MSC-SSCT was higher after SSCT, litter sizes after SSCT and MSC-SSCT were similar, and significantly reduction of expression of DNMT3A and H4K5ac in transplanted males, but the normal pattern in donor-derived offspring	(34)

Table 2: Continue

Type of stem cell	Other treatments	Animal model	Outcome	Reference
BMSCs (GFP^+)	NA	Mouse	Survival of stem cells within the seminiferous tubules, some with Sertoli cell appearance expressing FSH receptor, some expressing P450scc with appearance of spermatogonia or spermatocytes expressing VASA, treatment of azoospermia	(65)
BMSCs, SSCs	Busulfan testicular gonadotoxicity infertility model	Mouse	showed that BMSCs had no protective effect on fertility after chemotherapy, while after transplantation of SSCs spermatogenesis was observed in 83% of the injected testes	(66)
AFSCs	NA	Rat	Successful homing of AFSCs over the basement membrane of the injured seminiferous tubules, attenuation of degenerative and oxidative changes, re-expression of PCNA in the germ cells, resumption of spermatogenesis, re-appearance of spermatozoa	(41)
PSCs	NA	Mouse	Increase in expression of PCNA of KI67, decrease in apoptotic gene expression levels (*γ-H2AX*, *BRCA1*, *PARP1*), improved semen parameters, increased testosterone levels and testis size, promoting autophagy, protecting against oxidative stress, decrease in testicular oxidative damage, restoration of disrupted spermatogenesis	(42)
USCs	USC-exos	Mouse	Increased spermatogenic gene expression levels (*Pou5f1*, *Prm1*, *SYCP3*, and *DAZL*) and protein UCHL1, restoration of endogenous spermatogenesis, treatment of azoospermia	(43)
AMSCs	NA	Mouse	Restoration of spermatogenesis, elevated testosterone levels, enhanced testicular weight, size, and semen parameters, increased cell proliferation, ameliorated cell apoptosis, repressed oxidative damage, augmented oxidative defense, rise in the expression level of GCS genes (*Dazl*, *Ddx4*, and *Miwi*) and the meiosis genes (*Scp3*, *Cyclin A1*, and *Stra8*)	(44)

AdSCs; Adipose tissue-derived stem cells, AdSCs-CM; Adipose tissue-derived stem cells conditioned medium, AFSCs; Amniotic fluid-derived stem cells, AMSCs; Amnion mesenchymal stem cells, BMSCs; Bone marrow-derived stem cells, BMSCs-CM; Bone marrow-derived stem cells conditioned media, FSH; Follicle-stimulating hormone, GFP^+; Green fluorescent protein-positive, MSCs; Mesenchymal stem cells, NA; Not available, OCT4; Octamer-binding transcription factor 4, P450scc; Cytochrome P450 side chain cleavage enzyme, PCNA; Proliferating cell nuclear antigen, PSCs; Placental mesenchymal stem cells, RA; Retinoic acid, RFP; Red fluorescent protein, Scp3; Synaptonemal complex protein, SSCs; Spermatogonial stem cells, SSCT; Spermatogonial stem cell transplantation, SSEA-1; Stage specific embryonic Antigen-1, TFI; Tubular fertility index, TGFβ1; Transforming growth factor beta 1, UCPVCs; Umbilical cord perivascular cells, USCs; Urine-derived stem cells, USC-exos; Urine-derived stem cells exosomes.

When UCSCs and CD34/CD73-double-positive $CD34^+$/$CD73^+$ testicular stromal cells (TSCs) were used to counteract the deleterious effects of EDS-induced male infertility, restoration of spermatogenesis was noticed via improvement of testosterone level, expression of Leydig cell markers, like cytochrome P450, and polypeptide 1, 3-β-hydroxysteroid dehydrogenase (36, 80). Regarding transplantation of AFSCs in the treatment of the testicular torsion model of male infertility, recovery of spermatogenesis was observed which can be due to the impact of their secretory factors (81). Table 3 presents findings after injection of the various MSC sources into the testis of different animal models of male infertility, induced by methods excepting busulfan.

Cohort studies and case reports of male infertility treated by mesenchymal stem cells

In a cohort study, 105 males with impaired spermatogenesis who received allogeneic stem cell transplantation provided restoration of spermatogenesis (82). In patients undergoing chemo- and radiation therapy with impaired spermatogenesis, autologous bone marrow transplantation (BMT) showed repairing effects for spermatogenesis (83), and finally transplantation of BMSCs and UCSCs could similarly improve spermatogenesis (84). Transplantation of stem cells in these patients improved the hormonal profile of testosterone, follicle-stimulating hormone (FSH), luteinizing hormone (LH), prolactin, estradiol, and inhibin B which can describe their therapeutic effects (83, 84). In Table 4, the cohort studies and case reports regarding the treatment of male infertility using MSCs are presented based on autologous and allogenic sources of transplanted stem cells.

Clinical trials in male infertility treated by mesenchymal stem cells

Couto et al. (84) in their screening, during the years 2007-2017 revealed that the median UCSCs dose for local injection was 18.75 million; whereas the median UCSCs was 80 million in the systemic administration. Can et al. (85) in a systemic analysis of clinical trials showed that UCSCs transplantation was successfully used in repairing 25 male infertility suffering from oligospermia. Several factors were mentioned to affect the safety of these interventions, including a good manufacturing practice (GMP), as a gold standard to prevent employment of contaminated cells and inadequate handling or processing methods. So, under internationally recognized GMP conditions, clinical-grade stem cells are manufactured for cell transplantation purposes.

Table 3: Results of transplantation of MSCs from different sources into the testis of animal models of male infertility induced by the other treatment methods except busulfan

Type of stem cell	Induction method of infertility	Animal model	Outcome	Reference
BMSCs	Dox-induced testicular tissue toxicity	Rat	Decrease in testicular oxidative stress, reducing MDA level, increasing antioxidant capacity, recovering testicular atrophy and damages in spermatogenesis, reduction of diameter and germinative cell layer thickness of seminiferous tubules, restoring efficiency of reproductive system	(29)
BMSCs	Gonadotoxicity-induced models exposed to LN	Rat	Amelioration of testosterone level and semen quality, increase in superoxide dismutase, glutathione peroxidase and catalase levels, decrease in genomic DNA alteration and percentage of fragmented DNA, reversing tissue degeneration, necrosis, interstitial edema, reduction in spermatogonia and deformities in the morphology of testis, treatment of infertility	(37)
BMSCs	Electron beam irradiated infertility model	Rabbit	Improved spermatogenesis, recovery of infertility	(38)
BMSCs	$CdCl_2$-induced testicular injury	Rat	Improved pathological changes and expression of apoptosis-associated proteins (Bim, Bax, Cytochrome C, Caspase-3, active-Caspase-3, and AIF), increased Bcl-2, repairing testicular tissue injuries, restoration of fertility	(47)
BMSCs	$CdCl_2$-induced testicular injury	Rat	Increased SOD, LH and total antioxidants of serum and fructose of semen, modulated serum testosterone level, and reestablishment of spermatogenesis by differentiation into sperm, recovery of testicular function	(78)
BMSCs	Cisplatin-induced gonadotoxicity	Rat	Improvement of biochemical and histopathological changes, increase in MDA, TNF-α**,** BAX expression, iNOS, caspase-3, and p38-MAPK and seminiferous tubules atrophy, repairing testicular injury	(72)
BMSCs	$CdCl_2$-induced testicular damage	Rat	Decrease in testicular damages, repairing testicular damages, restoration of infertility	(73)
BMSCs	Dox-induced toxicity in the model of infertility	Rat	Decreased testicular oxidative stress, recovered testicular atrophy and damages of spermatogenesis, reduction of the diameter of seminiferous tubules, significant restoration of structural efficiency of the male reproductive system	(74)
BMSCs	Testicular torsion azoospermia rat model	Rat	Absence of germ cell-specific markers (Oct4, Vasa, and c-Kit), and sperm formation in biopsies	(75)
BMSCs	Traumatic testis rupture autoimmune infertility model	Mouse	Immunosuppressive effects on the production of ASA	(76)
BMSC-exos	Cyclophosphamide-induced testicular spermatogenic dysfunction	Rat	Enhanced cell proliferation, inhibited pathological changes, reduced apoptosis and phosphorylated levels of ERK, AKT, and p38MAPK proteins, a potential treatment for spermatogenic dysfunction	(32)
BMSCs co-transplantation with SSCs, TGFβ1	$CdCl_2$-induced testicular damage	Mouse	Increase in overall TFI and litter sizes, decreasing expression of DNMT3A and H4K5ac in germ cells, restoration of fertility	(71)
BMSCs anti-SCa-1 and CD105	Cyclophosphamide-induced azoospermia	Mouse	Improvement in androgen hormonal profile levels, resuming spermatogenesis, differentiation into germ cells and sperms, restoration of testicular function, treatment of azoospermic infertility	(46)

Table 3: Continue

Type of stem cell	Induction method of infertility	Animal model	Outcome	Reference
BMSCs	Cyclophosphamide-induced azoospermia	Rat	considerable restoration of sex hormone concentrations, enhancement in testicular tissue architecture, improvement of the spermatogenesis cycle	(77)
AdSCs	Cisplatin-induced testicular damage	Rat	Improvement of testicular architecture, increased testosterone level, immune reaction of CD-44, treatment of male infertility	(33)
AdSCs	$CaCl_2$ castration model of infertility	Rat	Improvement in the histological architecture of testicular tissue as well as sperm count and serum testosterone level, treatment of infertility	(36)
AdSCs	Testicular torsion-induced infertility model	Rat	Decreased testicular torsion-detorsion, improvement in sperm function and motility, increased expression levels of phosphoglycerate kinase 2 and glyceraldehyde-3-phosphate dehydrogenase-spermatogenic, activating Akt and GSK3, increase in glycolysis cascades and ATP production, reversing imbalance of glycolysis in sperm and testis, treatment of germ cell injury and infertility	(39)
AdSCs	Testicular torsion-induced infertility model	Rat	Improvement in Johnsen's score, preventing ischemic/reperfusion-induced intrinsic apoptosis, increased serum testosterone secretion, balancing FSH level surrounded Leydig cells by stem cells, rescued infertility	(79)
UCSCs CM-Dil-labeled	EDS-induced male rat hypogonadism model	Rat	Increased testosterone level, expression of Leydig cell markers cytochrome P450, family 11, subfamily A, polypeptide 1, 3-β-hydroxysteroid dehydrogenase treatment of male infertility	(36)
TSCs being CD34/CD73-double-positive $CD34^+$/$CD7^+$	Cavernous nerve crush injury model of infertility	Rat	Restoration of erectile function and fertility	(80)
AFSC	Ischemia/reperfusion injury of twisting the spermatic cord and testicular rotation on its long axis	Mouse	Restoration of normal sperm chromatin condensation, spermatogenesis parameters and histomorphometric organization of seminiferous tubules	(81)

AdSCs; Adipose tissue-derived stem cells, AFSC; Amniotic fluid stem cells, ASA; Antisperm antibody, Bax: B-cell lymphoma-2 Bcl-2 associated X protein, BMSCs; Bone marrow-derived stem cells, BMSCs-CM; Bone marrow-derived stem cells conditioned media, $CaCl_2$; Calcium chloride, $CdCl_2$; Cadmium chloride, Dox; Doxorubicin, EDS; Ethane dimethanesulfonate, FSH; Follicle-stimulating hormone, GSK3; Glycogen synthase kinase 3, iNOS; Inducible nitric oxide synthase, LN; Lead nitrate, MDA; Malondialdehyde, NO; Nitric oxide, SOD; Superoxide dismutase, SSCs; Spermatogonial stem cells, TFI; tubular fertility index, TGFβ1; Transforming growth factor beta 1, TNF-α; Tumor necrosis factor-alpha, TSCs; Testicular stromal cells, and UCSCs; Umbilical cord stem cells.

Can et al. (85) demonstrated that cell isolation and preparation procedures in 93 clinical trials still had a significant gap between the required quality conditions and the de facto status, while just 28% of the trials described standard GMP conditions in the manufacturing of clinical-grade stem cells. Evaluation of cell viability as an important issue in GMP protocols has just been explained in 19% of trials. The immunophenotypic characterization by flow cytometry, on the other hand, was used in 71% of trials. Also in 31% of trials, functional analysis for *in vitro* differentiation assays into three lineages have been undertaken to validate protocols of cell isolation and expansion. In 3% of trials, cell characterization was undertaken by cytokine production assay using an enzyme-linked immunosorbent assay (ELISA) before cell transplantation. So, it seems that the commercialization of MSCs can offer standard manufacturing methods and usage to be applied in clinical trials. As the quality of cells is very sensitive to transportation and storage conditions, local procurement of cells seems mandatory in diverse geographic regions especially when local cell banks provide the off-the-shelf MSCs, as noted in several clinical trials, especially from China.

Can et al. (85) in their investigation reported that 72% of the 93 trials had undertaken the safety of the interventions without any adverse events, and 29% of the interventions

found fever, local pain, headache, and dizziness that disappeared a few days post-transplantation. It is important to carefully monitor short-term signs when they are specific to cell injections or have no direct correlation to cell transplantation. More trials are expected to be published shortly as there are already many registered clinical trial databases. It seems that placebo-controlled, multicenter dose-escalation studies can promote the power of clinical research.

MSCs are generally cultured and expanded in Dulbecco's Modified Eagle's medium (DMEM), DMEM-Ham's F-12 or α-MEM culture media. These media are generally utilized for research purposes because they are frequently enriched with fetal bovine serum (FBS). In Table 4, clinical trials, cohorts and case reports regarding the treatment of male infertility are presented based on the source of stem cells.

Table 4: Cohort and case report studies and undertaken clinical trials using the various sources of MSCs in the treatment of male infertility

Type of study (Year)	Number of patients	Stem cell source, Route	Method	Outcome	Reference
Cohort (2021)	105 adult males with hypogonadism and impaired spermatogenesis	Allogeneic stem cell transplantation	Hormonal assessment (testosterone, FSH, LH, and inhibin B) utilizing IIEF-15 questionnaire and comparison with the general population	Restoration of testicular structure and function	(82)
Case report (2019)	A 36 years-old man after non-Hodgkin T-cell lymphoma treated with chemo- and radiotherapy	Autologous bone marrow	BMT after in mTESE	Showing an absolute restoration of fertility	(83)
Case series (2019)	25 patients with oligospermia	BMSCs, UCSCs	Hormonal profile of follicle-stimulating hormone, luteinizing hormone, prolactin, estradiol, testosterone, and findings of semen analysis	Improvement of the emerging evidence contributing to solve problems related to male infertility	(84)
Clinical trial (NA;2013-2015)	10 adult Kleinfelter syndrome patients, in Egypt	Bone marrow	Injection into the testicular tubules and testicular artery, Semen analysis, Hormonal profile	Completed, NA	NCT02414295
Clinical trial (phases I and II;2012-2017)	100 adultazoospermic patients, Egypt	Bone marrow	Injection into rete testis, hormonal assessment	Fertility improvement	NCT02025270
Clinical trial (NA; 2013-2016)	20 child and adult azoospermic patients, in Egypt	Bone marrow	Intra-testicular artery and inside tubules injection, Semen analysis, hormonal assessment	Recruiting	NCT02008799
Clinical trial (phases I and II; 2014-2015)	60 adult azoospermic patients, Egypt	Bone marrow	Injection into testis, hormonal profile, testicular biopsy	Recruiting	NCT02041910
Clinical trial (phases I and II;2015-2020)	50 adult azoospermic patients, Jordan	Bone marrow	Intra-testicular injection, investigating different germ cells in testicles, assessment of testicular morphology, sexual function	Recruiting	NCT02641769
Clinical trial (phase II;2018-2020)	40 adult azoospermic patients, Russia	Adipose tissue	Intra-testicular injection, sperm concentration in ejaculate, spermatozoa in testicular biopsy, hormonal profile	Recruiting	NCT03762967

BMT; Bone marrow transplantation, BMSCs; Bone marrow stem cells, LH; Luteinizing hormone, mTESE; Microdissection testicular sperm extraction, NA; Not available, and UCSCs; Umbilical cord stem cells.

Limitations of using mesenchymal stem cells in the treatment of male infertility

In recent years in modern science and medicine with the development of research on stem cells, increasing evidence was provided describing MSCs as a potential source in the treatment of male infertility. The advances in stem cell biology revealed the 'promiscuity' of MSCs to differentiate, not only into somatic lineages but also into gametic lineages and treatment of male infertility. *In vitro* models were also developed for spermatogenesis and drug screenings to assess gametogenesis. MSC treatments for infertility were divided into two major groups, including the direct transplantation of stem cells or their paracrine factors into reproductive organs, and the *in vitro* differentiation of stem cells into germ cells or gametes and their transplantation (85). In animal models, these therapeutic strategies were found to improve the reproductive potential of the tested animals but there is still few evidences in humans to reveal their conventional use based on the complexity of explored biological processes, the unavailability of proper materials, and ethical considerations. Evolutionary divergence noted in pluripotency among animals and humans still needs caution, when extrapolating the data obtained from murine models to safely apply them to clinics for humans (84, 85).

It seems that more clinical trials based on larger populations and long-term periods are needed to determine the relevance of stem cell therapy, including its efficacy in translational infertility medicine, since a small sample size and a low-quality method can lead to different outcomes. As the storage of stem cells is controversial, the patient is unable to provide his consent or truly understand the implications of the methods. In addition, many technical barriers still exist for most of the stem cell protocols and a well-defined standardized reproducible protocol for storage seems necessary before the use of cell therapies in the clinical landscape. The storage situations in various laboratories for lyophilization, cold chain, and transportation are different which can impact the study results. Moreover, senescent (non-functional) cells can influence the activity of the surrounding healthy cells by releasing several paracrine factors which should be avoided for clinical use to keep the treatment potential of the stem cell batch (84, 85).

Differences in MSC sources, injected cell number, times the cells were transplanted, route of administration, intervals between injections, differences in cell culture laboratories that apply various methods to isolate and purify stem cells, induction method of infertility, type of animal model, assessment methods, and follow-up time were also reported as important factors which can affect survival of the transplanted cells. The donor site for cell isolation can influence proliferation and differentiation potentials of the isolated MSCs which should be noted when cell transplantation is targeted. The genetic stability of transplanted cells is also of great importance and it should be monitored as any acquired mutation can pass to the next progeny. So, the stem cells need to be properly optimized and controlled to prevent any unnecessary cell growth and any probable infection. Furthermore, the intensity of clinical adverse effects can be ignored easily, if a "likely to predict efficacy" is included (85). As there are too many culture media which are specifically designed for human MSCs, there is a strict need for serum-free supplements to avoid using animal products during cell propagation, even if their formulations are expensive (86).

Couto et al. (84) in their screening (between the years 2007 and 2017) found that the cell manufacturing data was often lacking from the published reports. Given the missing variation in the outcomes and the absence of data about manufacturing, they could not identify any trends between outcomes and manufacturing. It should be mentioned that there might be many biases toward the publication of positive results in the literature, as positive findings are more easily and quickly published than negative and null findings (85). Despite these limitations, cell therapies utilizing MSCs remain a tempting strategy to overcome current obstacles in male infertility, because they can lead to regeneration in tissue pathologies. It should be mentioned that some ethical issues may be confronted when transplantation of MSCs is targeted for the treatment of infertility, as MSCs alone may sometimes have a meiotic block during differentiation (8). This may result in a lack of differentiation to SSCs (79). However, well-defined reproducible protocols are necessary for humans to confirm the efficacy of these cells to treat male infertility (87).

Conclusion

The results derived from the transplantation of MSCs in the treatment of male infertility seem encouraging and reveal the safety and efficacy of these cells to recover spermatogenesis; eventhough there is still a need for more stem cell research and clinical application of MSCs in the treatment of male infertility. Undertaking more well-defined, standardized and reproducible protocols, enrolling larger sample sizes and longer follow-up periods can verify the relevance of MSC transplantation in the restoration of spermatogenesis and treatment of male infertility. It seems that developing and utilizing stem cell transplantations, exosomes, scaffold delivery systems and 3D culture methods may open a new window in getting the most benefits from cell therapy in the treatment of men infertility.

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Authors' Contributions

D.I., D.M.; Drafted, Edited and Confirmed the manuscript. D.M., F.K.-B.; Reviewed and Edited the manuscript. All

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No Association between Single-Nucleotide Polymorphisms of The *S1PR1* Gene or Interleukin-17 Levels with Fingolimod Response in A Small Group of Iranian Relapsing-Remitting Multiple Sclerosis Patients: A Case-Control Study

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Abstract

Objective: Multiple sclerosis (MS) has a multi-factorial etiology involving genetic factors. Fingolimod (Gilenya ®, FTY720) modulates the G-protein-coupled sphingosine 1-phosphate (S1P) receptors, *S1PR1*, 2, 3, 4 and 5. Variation in the human *S1PR1* coding sequence results in heterogeneity in the function of the receptor. Interleukin-17, producing $CD4^+$ T cells, tends to be increased after treatment with Fingolimod. The aim of the study was to investigate single-nucleotide polymorphisms (SNPs) in the *S1PR1* gene or interleukin-17 (IL-17) levels in a small group of Iranian relapsing-remitting MS patients treated with Fingolimod.

Materials and Methods: In this case-control study, the genomic DNA of 94 MS patients treated with Fingolimod was extracted and Sanger sequencing was performed on polymerase chain reaction (PCR) products to detect variants in the *S1PR1* gene. Quantification of IL-17 from the serum of the patients was performed using a commercially available enzyme-linked immunosorbent assay (ELISA).

Results: Among 94 relapsing-remitting MS patients treated with Fingolimod, 69 (73.4%) were responders and 25 (26.6%) were non-responders. There were four novel and five common SNPs in the *S1PR1* gene and no significant association between SNP genotype and drug response was detected. In a subset of 34 patients, there was no significant difference in IL-17 serum concentrations before or after treatment and no association with *S1PR1* polymorphisms was determined.

Conclusion: This study is the first in Iran to investigate association between SNPs of the *S1PR1* gene or IL-17 levels with fingolimod response in a small group of Iranian relapsing remitting MS patients. There was no association with *S1PR1* gene SNPs or IL-17 levels before or after treatment.

Keywords: Fingolimod, Interleukin-17, Multiple Sclerosis, Polymorphism, Sphingosine 1-Phosphate Receptor

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Introduction

Multiple sclerosis (MS) is an inflammatory demyelinating and neurodegenerative disease of the central nervous system (CNS) with a multi-factorial aetiology involving an interaction of environmental (1) and genetic factors (2).

Sphingosine-1-phosphate (S1P) is a pleiotropic bioactive lipid. It is a crucial regulator of many physiological processes. S1P is produced by sphingosine kinase 1 or 2 (sK1, sK2) initially in red blood cells, platelets and endothelial cells (3). S1P up-regulates neurotrophic gene expression, which protects hippocampal neurons against excitotoxic cell death (4). The function of S1P is mediated by five specific G-protein coupled receptors (GPCRs) named S1PR1 to S1PR5 (S1P receptors 1 to 5) that bind to S1P and dihydro-S1P with high affinity (5).

The *S1PR1* gene is on chromosome 1p21 and it is expressed in a wide variety of cells (6). It regulates the trafficking of T and B lymphocytes (7) and the other haematopoietic cells including natural killer (NK) T-cells, dendritic cells, macrophages, neutrophils, haematopoietic progenitors, mast cells and osteoclasts (8). It is also involved in vascular development and maintenance

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(9). S1PR subtypes have different G-protein coupling, and their associated downstream signalling pathways are implicated in cell proliferation, survival, migration, and neural cell communication (10). Inhibition of S1PR signalling prevents dendritic destabilization and denervation-induced dendrite loss (11).

Variation in the human *S1PR1* coding sequence has been demonstrated to result in heterogeneity in the function of the receptor and impact on the interaction of S1PR1 with Fingolimod. Non-synonymous single nucleotide polymorphism (SNP), Arg^{120} to Pro, failed to transmit S1P-induced intercellular signals, such as calcium increase and activation of P44/42 MAKPK and Akt. Another mutation (Arg^{13} to Gly) was demonstrated to be protective against coronary artery disease in high cardiovascular risk populations, while the patients carrying this mutation had a significantly lower percentage of multi-vessel coronary obstruction (12). Reactive astrocytes in MS lesions, when cultured under pro-inflammatory conditions, strongly express S1PR1 and 3 (13).

Nagahashi et al. (14) found a mechanism involving the SphK1/S1P/S1PR1 axis Associated with obesity, inflammation, breast cancer progression and metastasis. Overexpression of S1PR1, S1PR2 and S1PR3 in bladder tumour, suggested that the S1PR profile in tumour biopsies was a promising marker in the diagnosis of bladder carcinoma (15).

Fingolimod is an orally administered drug applied for relapsing-remitting MS (RRMS). Fingolimod is a successful therapy for RRMS which decrease relapses, disability progression, new brain lesions and loss of brain volume (16).

Fingolimod has been reported to modulate the B cell cytokine profile, increasing the ratio of transforming growth factor-beta (TGF-β) and IL-4 to pro-inflammatory cytokines (17). The increased ratio of regulatory B cell subsets may be involved in influencing the cytokine environment and disease progression. Inhibition of S1PR signalling may prevent dendritic destabilization and denervation-induced dendrite loss (11). It also protects from neuro-inflammation by blocking the effect of S1PR1 expression in astrocytes which helps the blood brain barrier (18). Kim et al. (19) demonstrated that prolonged pFTY720 binding to S1PR1 triggered receptor internalization and degradation *in vivo*. Moreover, FTY720 had protective effects on oligodendrocytes.

Among 381 MS patients who were classified according to the no evidence of disease activity-3 (NEDA-3) criterion at 2 years, Ferrè et al. (20) found a genetic model containing 123 SNPs which could predict Fingolimod response with an area under the ROC curve (AUROC=0.65 in the independent test set. By adding clinical data, the model accuracy was improved to AUROC=0.71, meaning that combining clinical and genetic data, by applying machine learning methods, was beneficial to predict response to Fingolimod.

Interleukin-17 (IL-17) belongs to a family of cytokines, including IL-17A, IL-17B, IL-17C, IL-17D, IL-17E and IL-17F. Studies in MS patients demonstrated that IL-17 levels were particularly increased during relapse time, both in the blood and the CNS (21). IL-17 was involved in ectopic lymphoid follicle formation in the CNS (22).

In a model with phosphorylation-deficient receptors of the *S1PR1* gene, mice developed severe experimental autoimmune encephalomyelitis, as a result of the production of autoreactive T helper 17 cells (Th17) in peripheral blood and nervous system with the ability to produce IL-17 (23), while this mechanism may be pathogenic in MS. In an Iranian population, mRNA levels of *IL-17* have been reported to be significantly increased in MS patients. mRNA levels of *S1PR1* and *IL-10* were also significantly lower compared to the healthy controls (HCs) (24). Li et al. (25) found that MSCs-Exos reversed the increased Th17/Treg in Aplastic Anaemia $CD4^+$ T cells, as previously reported. A recent study showed that expression of SMAD7 and S1PR1 in $CD4^+$ T cells in peripheral blood were biomarkers of MS and Crohn's disease (26).

In this study, we investigated the polymorphism of the *S1PR1* gene. Moreover, we studied the association of *S1PR1* gene polymorphisms and levels of IL-17 before and after treatment with Fingolimod, in a small group of Iranian relapsing-remitting MS patients.

Materials and Methods

Subjects

In this case-control study, a total of 94 MS patients (69 female, 76.7%, and 21 male, 23.3%), treated with Fingolimod (0.5 mg orally once daily), were recruited from the Research Institute of Neurological Diseases, Imam Khomeini Hospital, Tehran University of Medical Science (Tehran, Iran). All of the MS patients were diagnosed according to McDonald criteria (27). The responder patients in this study referred to evidence of delayed disability progression, decreased deterioration from baseline in the Extended Disability Status Scale score and/or reduced MRI markers of disease progression (16). The Ethical approval for this study was granted from the Institute of Research Review Board at Tehran University of Medical Sciences, Tehran, Iran (91-02-24-1823-66824).

DNA extraction and *S1PR1* polymerase chain reaction

The genomic DNA of patients was extracted from peripheral blood cells by QIAamp DNA Blood Mini Kit (Qiagen, USA) according to the manufacturer's instructions.

The primers listed in Table 1 were designed to amplify *S1PR1* introns, exons and flanking sequence in the 5′ and 3′ UTR regions of the gene, in addition to overlapping along the sequence of the *S1PR1* gene containing untranslated region, intronic, and exonic regions (Fig.1). *S1PR1* gene (NM-001400) was genotyped in MS patients (Ensemble genome browser: Transcript: *S1PR1*-001 ENST00000305352, Chromosome 1: 101702444-101707074 forward strand). This gene has two exons, one coding exon, and one intron; the length of exon one is 163 bps, intron 1153 bps, and exon two is 1377 bps. The transcript length is 2909 bps and the translation length is 382 residues).

Polymerase chain reaction (PCR) was performed as follows: 200 ng of genomic DNA were added to the final volume of 10 µl mastermix with final concentrations as follows: 0.25 µM of each dNTP, *Taq* DNA polymerase 0.05 U/µl, PCR buffer 10x; concentration of each primer was 1 µM for *S1PR1-1, -2, -3, -4, -5, -6* and *-7*, but in *S1PR1-5* primers, concentration for each primer was 0.8 µM.

Thermocycling conditions for the primer pairs *S1PR1-1* were as follows: initial denaturation for 5 minutes at 95°C, followed by two cycles at 95°C for 30 seconds, annealing at 69°C for 30 seconds, extension at 72°C for 45 seconds, followed by two cycles at 95°C for 30 seconds, annealing at 67°C for 30 seconds, extension at 72°C for 45 seconds, followed by 45 cycles at 95°C for 30 seconds, annealing at 65°C for 30 seconds, extension at 72°C for 45 seconds and a final extension at 72°C for 5 minutes. Variations in the touchdown PCR conditions were as shown in Table S1 (See Supplementary Online Information at www.celljournal.org) for the other primer pairs.

The amplified products were analysed by electrophoresis using a 1.8% (w/v) agarose gel containing (5 µg/µl) SafeView dye (NBS Biological, UK) and visualized under ultraviolet light (G:Box Gel Image Analysis Systems, UK).

Table 1: The primer sequences used for *S1PR1* genotyping

Primer name	Primer sequence (5´-3´)	Amplicon size (bp)
S1PR1	F1: TGGCTCTTTCCCTGACTCT	577
	R1: AAACATACTCCCTTCCCGCA	
	F2: CCAAGAAATTCCACCGACCC	480
	R2: GCTCCGAGTCCTGACCAAG	
	F3: CTGTCCAGCTGCTCCACC	497
	R3: CTGCGGCTGAATTCCATGC	
	F4: GGCAAATTCAAGCGACCCAT	466
	R4: TTAGTCTTTGAGGAGGGGCC	
	F5: GGTCTAGCATTGTCAAGCTCC	470
	R5: GAGAGGAAGGATCCTGGCTA	
	F6: GCTGAGGCCAAAGTTTCCAT	498
	R6: GCTTTTAAGGGCACAAGAGGG	
	F7: TCATTTCAAGCAACAACATGGT	493
	R7: CCAAAGAGCTACACAATCCAGT	

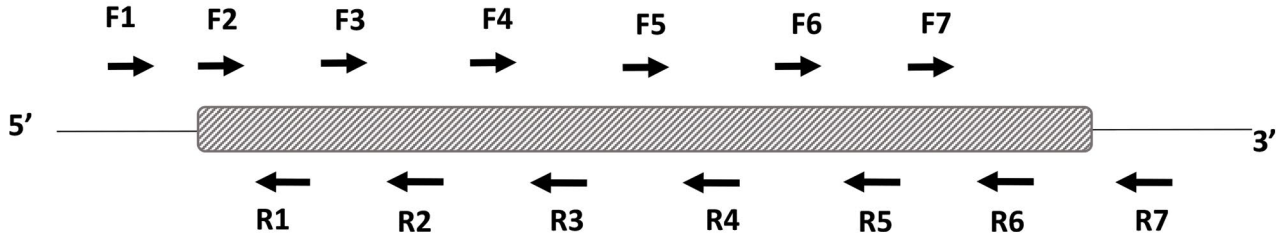

Fig.1: Overlapping primers along the sequence of *S1PR1* gene containing untranslated region, intronic, and exonic regions.

Direct Sanger sequencing of polymerase chain reaction products

Briefly, PCR products were purified using ExoSAP, containing Exonuclease I, (New England BioLabs, USA) and Shrimp Alkaline Phosphatase (USB Products Affymetrix, USA). Two µl of ExoSAP stock (containing 7.5 units Exonuclease I and 12.5 units Shrimp Alkaline Phosphatase) was added to 5 µl of PCR product. The reaction was incubated at 37°C for 40 minutes and then at 95°C for 5 minutes to deactivate the enzymes. 1 ng/µl (per 100 bp) of the PCR product from the clean-up step and 3.2 pmol/µl of sequencing primer (forward or reverse) were sent to Source Bioscience for Sanger sequencing (Cambridge, UK).

IL-17 detection in patient sera

Detection of IL-17 in patient sera was performed using the IL-17 Cytoscreen kit (Invitrogen, USA), according to the manufacturer's instructions. Absorbance was measured at 450 nm, 405 nm, and 630 nm using an ELISA reader (Reader type ELx808; Bio Tek, USA) for both standard curve and experimental samples. IL-17 concentration in each experimental sample was calculated using the standard curve.

Statistical analysis

Statistical Package for the Social Sciences (SPSS, version 22, IBM Corp., USA) was used for statistical analysis. The Chi-Squared test and Fisher's Exact test were used for qualitative data and the paired t test for quantitative data. Allele frequencies of *S1PR1* were tested for Hardy-Weinberg equilibrium (HWE) using the chi-squared test. The Hardy-Weinberg testing was carried out using an online calculator (Michael C., 2008). $P<0.05$ were considered statistically significant. Odds ratios (OR) were calculated and given with 95% confidence intervals (95% CI).

Results

Age, sex, and disease duration

Among the 94 relapsing-remitting MS patients treated with Fingolimod, 69 (73.4%) were responders and 25 (26.6%) were non-responders (Table S2, See Supplementary Online Information at www.celljournal.org).

There was no significant association between the gender of MS patients treated with Fingolimod and their response (whether they were responder or non-responder; data not shown).

S1PR1 genotyping data

Sanger sequencing

Among the 94 MS patients, three common variants (frequency ≥ 5%), two uncommon variants (frequency >1% and <5%) and four novel SNPs were detected by Sanger sequencing. These results are shown in Table 2. The agarose electrophoresis images are shown in Figure 2. The NOVEL1 and NOVEL2 SNPs were found in responders and NOVEL3 and NOVEL4 in non-responders. All novel variants were located in the 3′ UTR of the *S1PR1* gene. Sequencing electrophorograms of rs114115083 and novel variant 1, in MS patients are shown in Figures 3 and 4.

Table 2: Chromosome position, SNP name, alleles, type of variant and minor allele frequency of the five known and four novel *S1PR1* SNPs ordered by location in the gene

Position in chromosome	SNP	Alleles	Type of variant	Minor allele frequency*
101238940	rs3737578	T/C	5-prime UTR	11%
101238976	rs3737577	G/T	5-prime UTR	15%
101239016	rs61734752	C/A	Missense	2%
101240681	NOVEL3	G/T	3-prime UTR	-
101240690	rs114115083	T/C	3-prime UTR	1%
101240850	rs55872049	C/A	3-prime UTR	5%
101241054	NOVEL4	G/T	3-prime UTR	-
101241183	NOVEL1	A/G	3-prime UTR	-
101241436	NOVEL2	T/A	3-prime UTR	-

SNP; Single-nucleotide polymorphisms, UTR; Untranslated region, and *; Frequency of the second most frequent allele in 1000 Genome project phase 3 combined population.

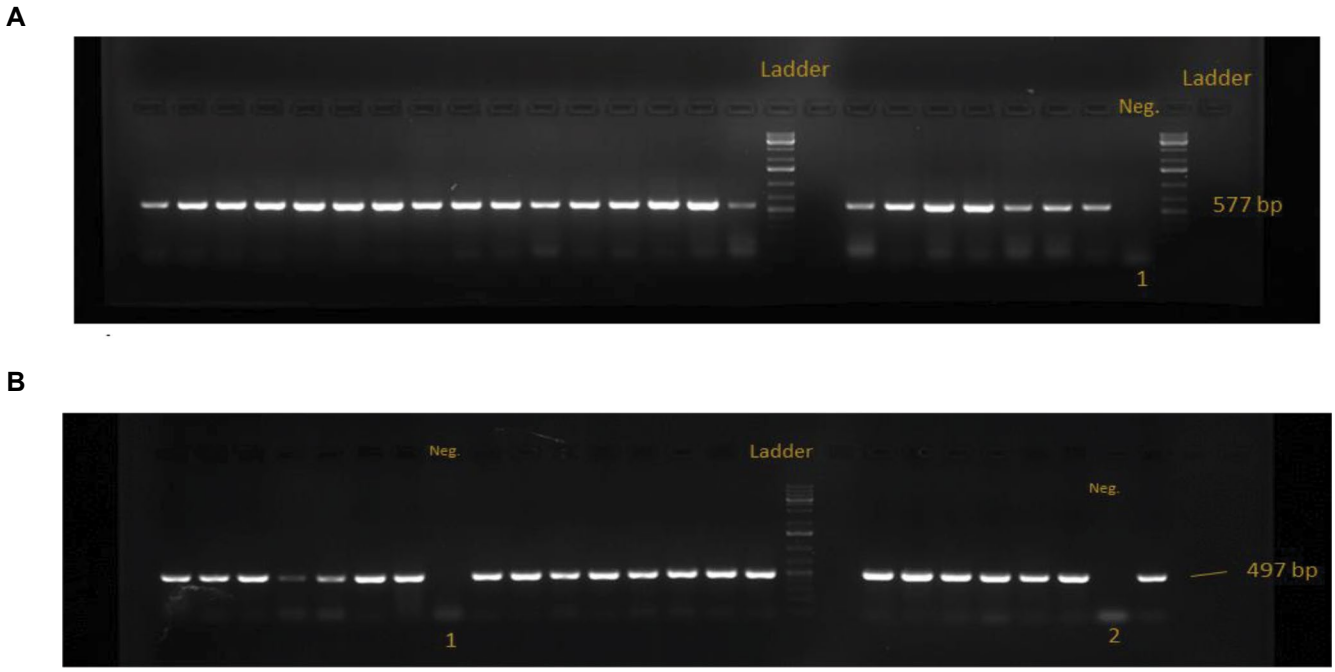

Fig.2: Agarose gel electrophoresis image of *S1PR1* gene amplicons. Amplicon sizes are as following: **A.** Primer 1: 577 bp and **B.** Primer 3: 497 bp. Neg.; Negative control marked as lane 1 or 2.

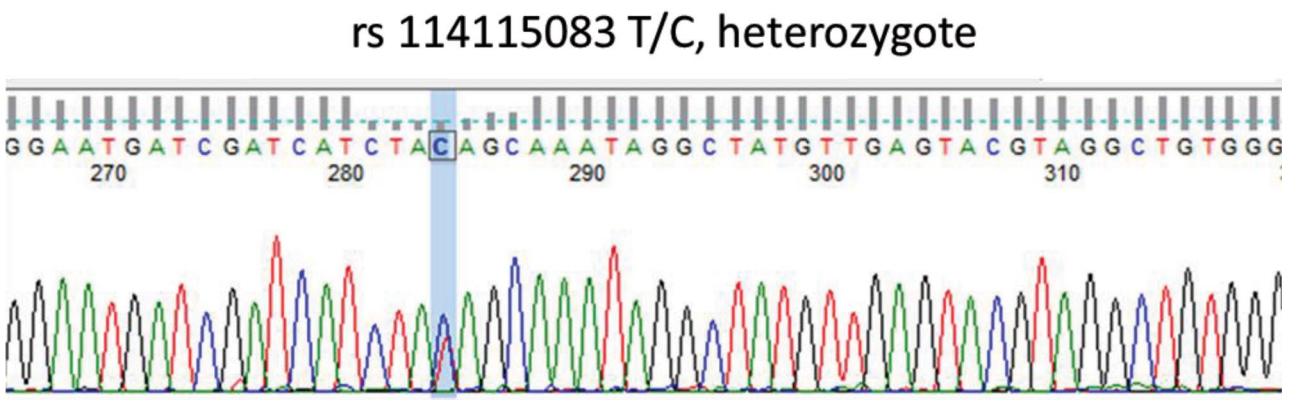

Fig.3: Sequencing electropherograms of rs114115083 in multiple sclerosis (MS) patients. The nucleotide "T" was substituted by "C" in this variant.

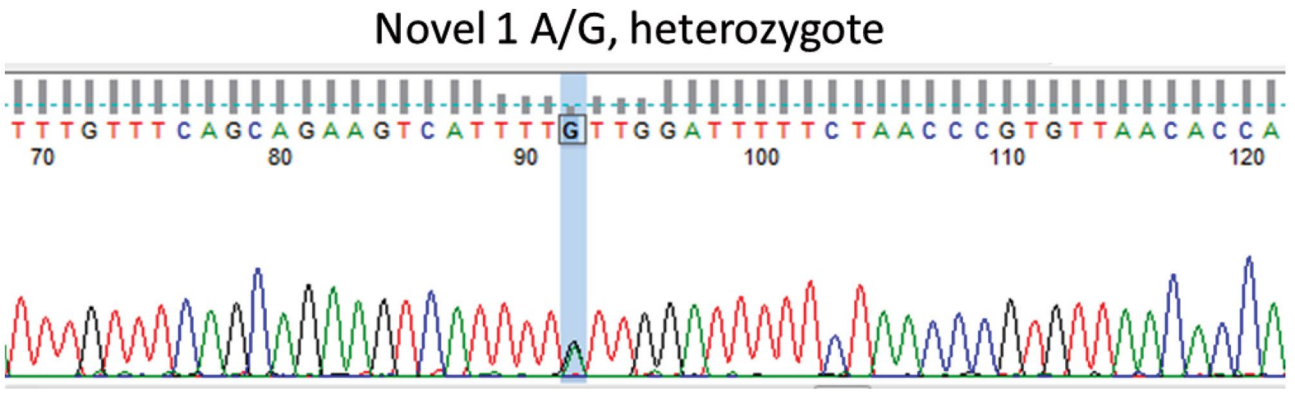

Fig.4: Sequencing chromatogram of novel variant 1 in multiple sclerosis (MS) patients. The nucleotide "A" was substituted by "G" in this variant.

Statistical analysis of *S1PR1* genotyping data

Quality control of rs3737577 and rs3737578 variant genotyping

HWE calculation gave P values of 0.126 and 0.851 for rs3737577 and rs3737578 respectively. This calculation was not possible for the rarer variants.

Statistical analysis of known and novel variants

The results of testing for the association of variants with Fingolimod response are shown in Table S3 (See Supplementary Online Information at www.celljournal.org). No significant association was observed for any variants (P>0.05 in all tests).

Concentration of serum IL-17 in multiple sclerosis patients treated with Fingolimod

Sera, collected from the patients, were obtained before and after treatment with Fingolimod. The mean and median of IL-17 levels in the serum of both responder and non-responder groups before and after treatment are shown in Table S4 (See Supplementary Online Information at www.celljournal.org).

There was no statistically significant difference (P<0.05) in IL-17 concentration between the patients before and after treatment with Fingolimod using a paired t test.

Discussion

The frequency of response to Fingolimod treatment in the MS patients was about three times more than non-responders (frequency of response was 73.4 vs. 26.6% for non-response). Although the frequency of females in the non-responder group (80%) was slightly more than in the responder group (75.4%), there was no significant association between gender and response to Fingolimod (P: 0.643, odds ratio: 1.306, 95% CI: 0.422-4.047).

Sanger sequencing identified four novel SNPs and five common SNPs. Two novel SNPs were found in the responder group and two in the non-responder group. Detected SNPs were in the 5′ UTR, and the 3′ UTR. Additionally, one SNP (rs61734752) was a coding region missense variant (C/A) while it was considered to be a benign substitution (28).

The common SNPs are not significantly associated with response to Fingolimod. However, there is likely to be insufficient statistical power to test for association in this study, so this cannot be ruled out for any of them. While the study is fundamentally underpowered, any smaller effects due to confounding would be undetectable, as they would be non-significant.

Moreno-Torres et al. (29) found that MS patients treated with Fingolimod displayed lymphopaenia together with a reduction in naïve T cell (TN), central memory T cells (TCM), memory B and natural killer (NK) bright cells and relative growth in effector memory T cell (TEM), terminally differentiated effector T cell (TEMRA), native B, transitional B, plasma blast, NK, and NK T cells (NKT) in the peripheral blood. They did not reveal difference in the expression levels of the genes coding CCR7, CD45RA, CD31, and PTK7 surface markers (CCR7, PTPRC, PECAM1 and PTK7) between the responder and non-responder patients before treatment.

Fingolimod applies a potent anti-inflammatory effect by reducing the proportion of cytokine producer T and B cells. This effect happened at both cellular and molecular levels, as Fingolimod improved a downregulation of genes, such as CD40L, CD 40, IRF4, CR2, IL-23A, CD2, IL-17A, and IL-17D. Likewise, the cytokine activity pathways were downregulated after treatment. In MS patients, a high proportion of NK bright cells has recently been associated with stable MRI. A higher percentage of these cells before treatment was associated with a good response, while they were reduced to a lesser degree after treatment in the responder compared to the non-responder patients. Fingolimod raised the proportion of NK cells. It also upregulated NCAM1 (CD56) and FCGR3A (CD16a) at the transcriptional level (29).

Fingolimod downregulated genes were involved in oxidative stress and suppression of stress-fiber formation, by the rhoA-ROCK1-NFKB pathway, showing that the anti-oxidant effect of Fingolimod was not related on innate immune activation (29). In the MS patients, lower serum levels of reactive oxygen species (ROS) were noticed in the patients treated with Fingolimod compared to the first-line-treated patient, and a relation between ROS levels and disease duration was shown (30). The decline in lymphocyte activation in response to Fingolimod may come up with a decrease in FOXP3 expression levels. Moreover, growth in the expression of IL-10, IL-10RA, IL-10RB, IL-15, and TNFRSF1A displayed a strong inductive effect of regulatory mechanisms in response to Fingolimod.

Upregulation of the apoptotic pathway implied that Fingolimod induced programmed cell death and it may contribute to lymphopenia. Overexpression of ADA2, SGK1, and BCL2L13 after six months of treatment in only responder patients, may suggest a differential effect of Fingolimod on the mechanisms of cell proliferation and differentiation (29). Tumour necrosis factor receptor super family member 13B (TNFSF13) and its ligand B cell activating factor have also been shown to contribute in the potential role of proliferation and differentiation of B cells in MS (31). Moreno-Torres et al. (29) found an important downregulation of IRF4 (a highly expressed molecule in activated B cells), CD27 (a memory B cell marker) and CXCR5, and a decline in the proportion of memory B cells after treatment. Moreover, a higher proportion of plasma blasts and overexpression of CXCL13 before treatment in the patients with NEDA response, showed that manipulation of B cell activation played an important role in the clinical response to Fingolimod. Immunoglobulin receptor *FCRL1* and *FCRL2* genes in the responder patients, despite the growth in the production of immunoglobulins, had a lower

activity of their receptors which is related to radiological activity in MS (32). This is in line with the fact that Fingolimod made a better effect in patients with a more inflammatory component. Moreover, Moreno-Torres et al. (29) determined a reduction in IL-2 producing cells and a downregulation of the IL-2RA gene in PBMCs of treated MS patients. Moreover, a decrease in IL-2 producing cells between NEDA and evidence of disease activity (EDA) patients was found, but no difference was noticed at the transcription level.

The study of Malhotra et al. (33) suggested that Fingolimod treatment may have benefit effects via inhibiting NLRP3 inflammasome activation in peripheral blood mononuclear cells in MS patients.

Hoffmann et al. (18) found that FTY-p induced neuroprotective factors, including leukaemia inhibitory factor (LIF), IL-11, and heparin-binding EGF-like growth factor (HBEGF). It can also suppress tumor necrosis factor (TNF) induced inflammatory cytokines BAFF and CXCL10. Moreover antiviral proteins like 2′-5′-oligoadenylate synthetase 2 (OAS2) and myxovirus resistance 1 (MX1).

Proschmann et al. (34) found people with MS (PwMS) treated with Fingolimod and HCs respond to acute exercise; they had a comparable temporal pattern of increased immune cell counts, immediately after exercise and a return to baseline levels within 1 hour.

Long intergenic non-coding RNA (Linc) 00513 has been recently reported as a novel regulator of the type 1 signalling pathway. Polymorphisms in the promoter region of Linc00513 (“G” for rs205764 and “A” for rs547311) have been associated with an overexpression of Linc00513 and growth in the downstream signalling activity of the type 1 IFN pathway (35).

In the Amin et al. (36) study, the MS patients carrying polymorphisms at rs547311 displayed a significantly higher disability score compared to the patients who carried the major allele. No significant difference was noticed in the other models of inheritance, suggesting that a single or double "A" allele(s) are equally harmful for a patient’s EDSS. Moreover, polymorphism at rs205764 showed to have no association with the EDSS. These findings are compatible with patients disability and the study which detected rs205764 and rs547311 as novel regulators of IFN signalling. Several other variants have been associated with differences in EDSS for MS patients, including rs17445836 in the interferon regulatory factor-8 gene, rs3087456 and rs4774 in class-II trans-activator gene, rs1049269 in transferrin gene, and rs1494555 in interleukin-7 receptor gene.

The 3′ UTR is the regulatory noncoding part of an mRNA containing a potential role in gene expression (37). The 3′ UTRs carry a special class of noncoding variants which may influence post-transcriptional and translational processes. Causal peripheral blood cis-expression Quantitative Trait Local (eQTL) presence is 4-fold enhanced in 3′ UTRs, a level comparing with that of promoter elements. Among the all tissues on the genotype-tissue expression project (GTEx), eQTLs in 3′ UTRs were shown to be 2-fold enhanced, which was the largest enhancement across all non-coding regions. Untranslated regions harbour the greatest enhancement of GWAS heritability (5-fold) of all non-coding classes, whereas transcription start sites, confirm an important role for post-transcriptional activities in human regulatory variation. Griesemer et al. (38) developed the Massively Parallel Reporter Assay for 3′ UTRs (MPRAu) to evaluate allelic expression differences for thousands of 3′ UTR variants simultaneously, in a high-throughput, accurate, and reproducible method. MPRAu tool functionally identifies 3′ UTR variants. It is applied to characterize 2368 3′ UTR variants that modulate transcript abundance among six cell lines. They developed MPRAu which is a strong predictive model of 3′ UTR function and characterized a novel way of 3′ UTRs regulation. They anticipate this model will be a common experimental paradigm to validate variants of unknown significance and rare variants in future. They found additional evidence applying 3′ UTR tiling and endogenous allelic replacement for three variants (rs1059273, rs705866, and rs34448361) with very important results to realize human disease and evolution.

The results in our study suggested that response to treatment with Fingolimod was not associated with *S1PR1* gene polymorphism in our study population. Further investigations with a greater sample size are required to thoroughly determine the effect of polymorphism in *S1PR1* on MS.

Serum levels of IL-17A and IL-17F in Iranian MS patients by comparing with HCs have a significant rise, moreover, a significant positive correlation of IL-17F serum levels with the number of relapses has been reported (39). However, our study did not find any significant differences in total IL-17 levels before and after treatment.

In addition, a significant decrease in IL-10, IL-27 and TGF-beta, has been reported but with no significant difference in IL-17 and IL-23 levels between the MS patients and healthy controls. This is in line with the results of our study in IL-17 serum levels. Higher IL-17, IL-19, and IL-4 producing CD^+ T cell percentages were reported in pre-treatment MS patients compared to the healthy controls, while the IL-17 producing CD^+ T cell percentages contribute to show a transient rise at 2 weeks of Fingolimod therapy (40). This suggested that the detection of variation in IL-17 levels may depend on the time serum sample collection from the patient after treatment.

Additional studies with larger sample sizes and multiple sampling time points should be the next stage to definitely confirm whether Fingolimod treatment affects the level of IL-17 in the control and MS patient sera.

Conclusion

This study is the first in Iran to investigate association between SNPs of the *S1PR1* gene or IL-17 levels with fingolimod response in a small group of Iranian relapsing remitting MS patients. The response to treatment was not associated with S1PR1 gene SNPs or IL-17 levels before or after treatment.

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Authors' Contributions

N.M.; Investigation, Writing the original draft, Review, Editing, and Visualization. P.S.; Validation, Editing, and Review. A.J.W.; Supervision, Review, Editing, and Validation. All authors read and approved the final manuscript.

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Effects of Idebenone on Rat Schwann Cells with Toxicity Induced by Hydrogen Peroxide: Assessment of Molecular, Apoptosis, and Oxidative Stress Parameters

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Abstract

Objective: Schwann cells are the main cells for myelination and regeneration of peripheral nerves. Idebenone is a synthetic antioxidant used to treat central nervous system diseases. The aim of the study is to determine whether idebenone can protect Schwann cells and increase cell activity under conditions of oxidative stress caused by hydrogen peroxide (H_2O_2) *in vitro*.

Materials and Methods: In this experimental study, Schwann cells were pre-treated with various concentrations of idebenone and H_2O_2; after determining the appropriate doses, the cells were treated with 10 μM idebenone for 48 hours and 1000 μM H_2O_2 for the last two hours. The malondialdehyde (MDA) level, and activity of superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) were assessed by ELISA. Cell viability was assessed by the MTT assay. Western blot analysis was conducted to determine the expressions of myelin protein zero (MPZ) and peripheral myelin protein 22 (PMP22), and expression ratio of the Bax/Bcl-2 proteins. The percentage of cell apoptosis was evaluated by annexin V staining using flow cytometry.

Results: Schwann cells under oxidative stress conditions caused by H_2O_2 and treated with idebenone had increased cell viability; increased SOD, CAT, and GPx activity; and increased expressions of the MPZ and PMP22 proteins. There was a decreased level of MDA, decreased expression ratio of Bax/Bcl-2 proteins, and a decrease in the percentage of apoptotic cells stained with Annexin V.

Conclusion: The appropriate dose of idebenone may improve both survival and function of Schwann cells exposed to H2O2 by reducing oxidative stress and apoptosis.

Keywords: Apoptosis, Idebenone, Oxidative Stress, Schwann Sells

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Introduction

Schwann cells are the main cells for myelin formation in peripheral nerves and are essential for the growth, regeneration, and function of these nerves (1). These cells effectively repair damaged peripheral nerves by producing growth factors and proteins necessary for myelination and axonal growth. Among the most important proteins produced by Schwann cells for myelination are myelin protein zero (MPZ) and peripheral myelin protein 22 (PMP22), which are directly related to remyelination and repair of peripheral nerves (2). When peripheral nerves are injured, the expression of these proteins increases for remyelination of damaged axons (3, 4).

Oxidative stress is a known factor that increases apoptosis. Under oxidative stress conditions, an imbalance occurs between the production of reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2) and antioxidants of body cells (5, 6). ROS attack intracellular proteins and nucleic acids, which leads to mitochondrial dysfunction and apoptosis (7).

Idebenone is a synthetic analogue of coenzyme Q10; although it has antioxidant properties, it is not obtained from natural materials. Idebenone provides electrons to reduce the effects of free radicals and supports mitochondrial respiratory chains to aid in ATP synthesis. In addition, idebenone affects the expression of mitochondrial complexes and can compensate for mitochondrial dysfunction (8). Idebenone is used to treat central nervous system diseases by repairing mitochondrial dysfunction. Its beneficial effects have been shown in neurodegenerative disorders such as Parkinson's and Alzheimer's diseases, and hereditary optic neuropathy (9, 10). In addition, the protective effect of idebenone on increasing bone marrow mesenchymal stem cell viability

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has been observed *in vitro* (11).

Although the antioxidant effects of idebenone have been studied in the repair of central nervous system diseases (12, 13), its effects on peripheral nerve damage and Schwann cells have not been investigated. Therefore, this study aims to determine whether the effects of idebenone could reduce apoptosis and increase both viability and function of Schwann cells under conditions of H_2O_2-induced oxidative stress *in vitro*.

Materials and Methods

Schwann cell culture

All procedures were approved by the Research Council of Semnan University of Medical Sciences, Semnan, Iran (IR.SEMUMS.AEC.1401.006). Newborn male Wistar rats (3-5 days old) were deeply anaesthetised by intraperitoneal injections of 80 mg/kg ketamine and 10 mg/kg xylazine. After opening the skin behind the thigh, the bilateral sciatic nerves were removed and cut into 1×1 mm^2 fragments, and then digested in 0.25% trypsin (Gibco, Germany) and 0.3 mg/ml collagenase type 1 (Sigma, USA) at 37˚C for 30 minutes. Then, 10% foetal bovine serum (FBS, Gibco, Germany) was added to terminate the digestion process, and the cells were centrifuged. Next, the cells were cultured in Dulbecco's Modified Eagle Medium with F-12 supplement (Gibco, Germany) that consisted of 10% FBS at 37˚C, 95% humidity, and 5% CO_2. After 24 hours, 5 µg/ml cytosine arabinoside (Sigma, USA) was added to the culture medium for 48 hours to purify the cells. Schwann cells were then exposed to the drugs (1).

Cell culture treatment

Schwann cells were exposed to different concentrations of idebenone (1, 2.5, 5, 10, 20, 40, and 80 µM, Sigma, USA) for 48 hours. Preliminary evaluations by the MTT assay showed that 10 µM idebenone had the most potent protective effects on cell proliferation; therefore, we continued the experiments with this dose. The Schwann cells were also exposed to H_2O_2 to evaluate the effects of idebenone under oxidative stress conditions. For this purpose, the half-maximal inhibitory concentration (IC_{50}) was determined by exposing the Schwann cells to various concentrations of H_2O_2 (125, 250, 500, 1000, 2000, and 4000 µM, Sigma, China) for two hours. Our results indicated that the IC_{50} was about 1000 µM. Therefore, the Schwann cells were exposed to 10 µM idebenone for 48 hours and 1000 µM H_2O_2 was added for the last two hours. The cells were equally divided into four groups: i. Control group: Schwann cells were cultured without exposure to the drugs, ii. Idebenone group: Schwann cells were cultured and exposed to 10 µM idebenone, iii.. H_2O_2 group: Schwann cells were cultured and exposed to 1000 µM H_2O_2, and iv. Idebenone+H_2O_2 group: Schwann cells were cultured and exposed to 10 µM idebenone and 1000 µM H_2O_2. The cells were washed three times and incubated with serum-free medium for 24 hours before the experiments were performed (14). All experimental procedures on the different groups of Schwann cells were repeated three times.

Cell viability (MTT assay)

Approximately 1×10^6 cells in 50 µl of culture medium were incubated in 96-well plate for 24 hours. Then, 500 µg/ml MTT was added to the wells and the plates were incubated for another three hours. The medium was removed and 100 µl of DMSO was added to completely dissolve the formazan crystals, and the results were read by an ELISA reader (BioTek, ELX800, USA) at 570 nm (15).

Flow cytometry assay

Flow cytometry assay was conducted to confirm the presence of Schwann cells and evaluate the percentage of apoptosis in these cells. In order to confirm the Schwann cells, we incubated the cells with fluorescence-labelled monoclonal antibody against an anti-S-100 antibody (Sigma, China). After washing, the labelled cells were analysed by a Becton Dickinson FACSCalibur flow cytometer (BD, USA) (16).

We assessed the apoptosis percentage of Schwann cells as follows. The cells were suspended and stained using an Annexin V-FITC Cell Apoptosis Assay Kit (Roche, Germany) according to the manufacturer's instructions. The stained Schwann cells were analysed by a Becton Dickinson FACSCalibur Flow Cytometer (BD, USA).

Measurement of oxidative stress biomarkers

We evaluated the amount of oxidative stress in the Schwann cells. The level of malondialdehyde (MDA) (pro-oxidant biomarker), and the activity of superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx), as antioxidant enzymes, were measured using assay kits (ZellBio GmbH, Germany) according to the manufacturers' instructions.

Western blot assay

The expression levels of MPZ, PMP22, Bax, and Bcl-2 proteins in the cultured Schwann cells were assessed by Western blot. The cells were lysed using radioimmunoprecipitation assay buffer (Sigma, USA). After electrophoresis, the proteins were transferred onto nitrocellulose membranes (Amersham Biosciences, Inc., USA) and blocked by using 5% skim milk. The membranes were then incubated overnight at 4°C with primary antibodies MPZ, PMP22, Bax, Bcl-2, and β-actin (1:1000, Santa Cruz Biotechnology, Inc., USA). After the primary antibody incubation, the membranes were treated with the secondary antibody, goat anti-rabbit conjugated with horseradish peroxidase (1:5000, Santa Cruz Biotechnology, USA). Immunoreactive bands were visualised by using a chemiluminescence detection kit (Bio-Rad, USA) and quantified with ImageJ software (17).

Statistical analysis

Data were analysed by one-way analysis of variance (ANOVA) and two-way ANOVA, followed by Tukey's post hoc test for multiple comparisons. All values are presented as mean ± SEM. P<0.05 was considered to be statistically significant.

Results

Determination of the drug concentrations

We used the MTT assay to determine the most effective dose of idebenone and IC_{50} value of H_2O_2 on Schwann cell proliferation. The mean of three repetitions of Schwann cells exposed to different doses of idebenone and H_2O_2 were analysed by one-way ANOVA. The results showed that the 10 and 20 µM doses of idebenone were significantly more effective compared to the control group (P=0.003 and P=0.015, respectively). The 10 µM was more favourable than the 20 µM dose; therefore, we chose the 10 µM dose for our analyses (Fig.1A). Although all doses of H_2O_2 significantly decreased Schwann cell viability compared to the control group (P<0.05), the IC_{50} for H_2O_2 was 1000 µM. Therefore, we chose this dose for our assessments (Fig.1B).

Viability and confirmation of Schwann cells

Subsequently, we conducted another MTT assay to evaluate Schwann cell viability in all of the groups. Addition of H_2O_2 significantly decreased Schwann cell viability compared to the control group (P<0.001). Cell viability in the idebenone group was significantly higher compared to the control group (P=0.036), and in the idebenone+H_2O_2 group, it was significantly higher compared to the H_2O_2 group (P=0.009). Figure 2A shows the results of two-way ANOVA assessment of Schwann cell viability after idebenone treatment ($F_{(1,8)}$=31.04, P=0.001), H_2O_2 treatment ($F_{(1,8)}$=639.4, P<0.001), and idebenone+H_2O_2 treatment ($F_{(1,8)}$=0.51, P=0.5).

Flow cytometry analysis was used to confirm S-100 protein expression by the Schwann cells after purification with cytosine arabinoside. The results demonstrated that most cultured Schwann cells expressed the S-100 protein (Fig.2B). Cultured Schwann cells in different groups are shown in the Figure 2C-F.

Oxidative stress biomarkers

The results showed that the addition of H_2O_2 significantly increased oxidative stress in the medium compared to the control group (P<0.001). The pro-oxidant biomarker MDA levels in the idebenone group were significantly lower than the control group (P=0.025), and they were significantly lower in the idebenone+H_2O_2 group compared to the H_2O_2 group (P=0.002). Figure 3 shows the two-way ANOVA analysis for the MDA biomarker after idebenone treatment ($F_{(1,8)}$=44.02, P<0.001), H_2O_2 treatment ($F_{(1,8)}$=528.6, P<0.001), and idebenone+H_2O_2 treatment ($F_{(1,8)}$=1.93, P=0.2).

Our assessment of the antioxidant biomarkers showed that idebenone significantly increased SOD (P=0.027), CAT (P=0.017), and GPx (P=0.047) compared to the control group. There was a significant increase in the idebenone+H_2O_2 group in SOD (P=0.004), CAT (P=0.011), and GPx (P=0.023) compared to the H_2O_2 group. Figure 3 shows the two-way ANOVA for SOD, CAT, and GPx after idebenone treatment ($F_{(1,8)}$=33.08, $F_{(1,8)}$=34.3 and $F_{(1,8)}$=24.5, P<0.001, P<0.001 and P=0.001, respectively), H_2O_2 treatment ($F_{(1,8)}$=461.06, $F_{(1,8)}$=369.3 and $F_{(1,8)}$=971.4, P<0.001, P<0.001 and P<0.001, respectively), and idebenone+H_2O_2 treatment ($F_{(1,8)}$=1, $F_{(1,8)}$=0.06 and $F_{(1,8)}$=0.13; P=0.35, P=0.81 and P=0.72, respectively).

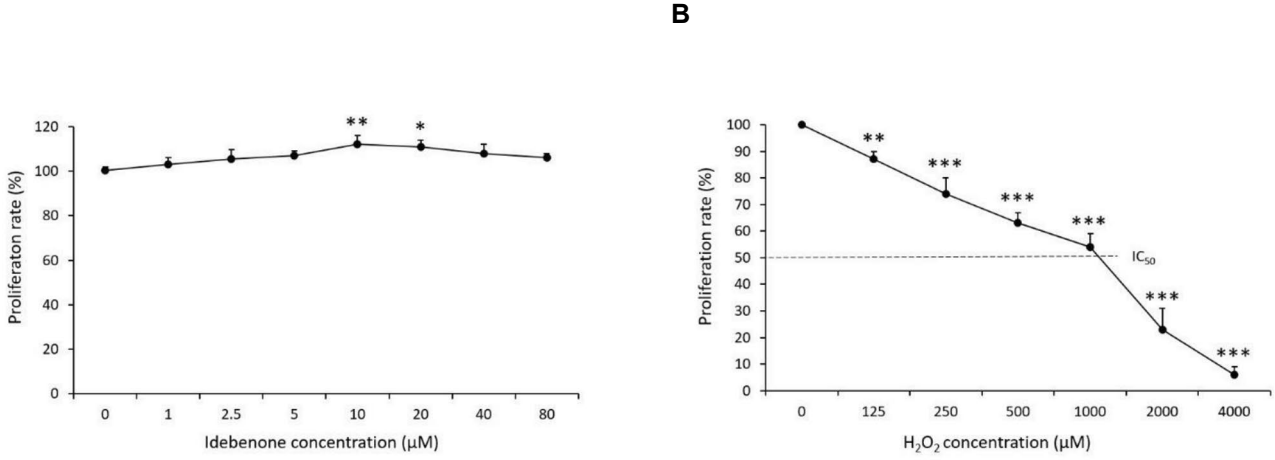

Fig.1: Effects of idebenone and H_2O_2 on Schwann cell proliferation. **A.** The most protective dose of idebenone is 10 µM after 48 hours of exposure. **B.** The half-maximal inhibitory concentration (IC_{50}) value of H_2O_2 is 1000 µM after two hours of exposure. *; P<0.05, **; P<0.01, and ***; P<0.001 vs. the control group.

Annexin V staining

The results of annexin V staining showed that H_2O_2 significantly increased Schwann cell apoptosis compared to the control group (P<0.001). The percentage of Schwann cell apoptosis in the idebenone group was significantly lower than the control group (P=0.002), and in the idebenone+H_2O_2 group, it was significantly lower compared to the H_2O_2 group (P<0.001). Figure 4 presents the results of two-way ANOVA for annexin V staining for Schwann cell apoptosis after idebenone treatment ($F_{(1,8)}$=186.1, P<0.001), H_2O_2 treatment ($F_{(1,8)}$=2648.8, P<0.001), and idebenone+H_2O_2 treatment ($F_{(1,8)}$=31.25, P=0.001).

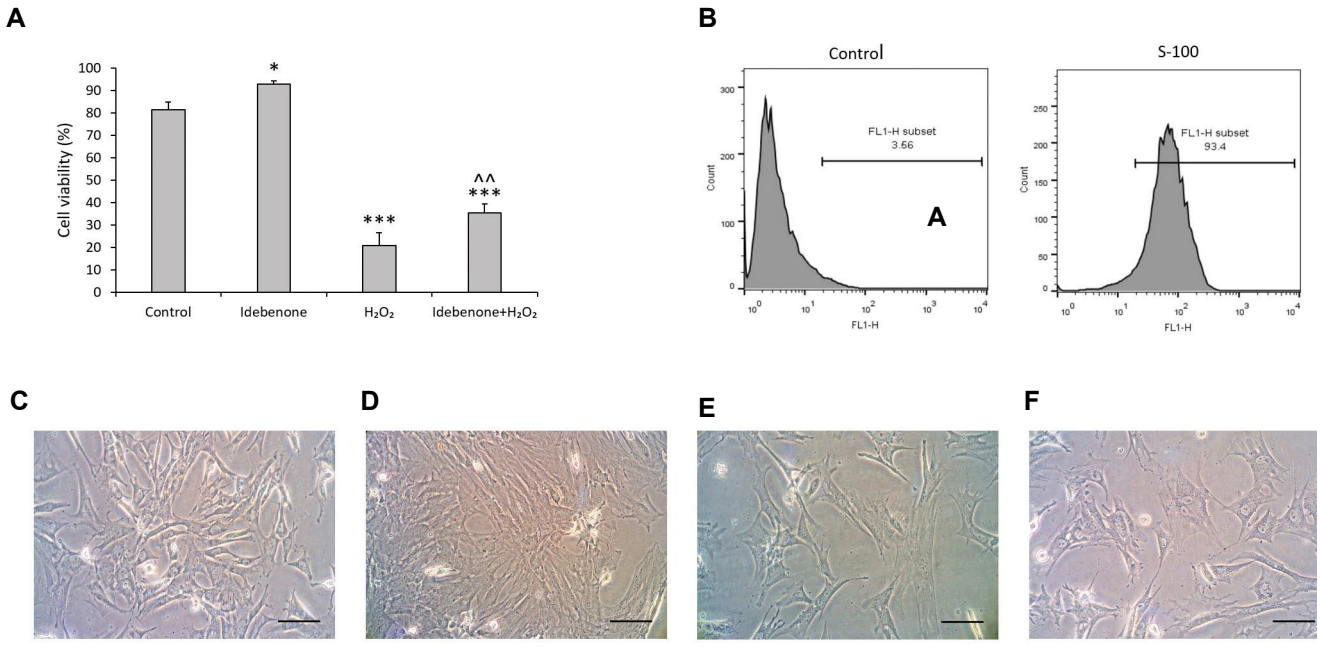

Fig.2: Percentage of viability and S-100 protein expression in Schwann cells. **A.** Effects of idebenone and H_2O_2 on Schwann cell viability. **B.** The results of flow cytometry show that most Schwann cells express the S-100 protein. **C.** Images of cultured Schwann cells in the control, **D.** Idebenone, **E.** H_2O_2, and **F.** Idebenone+H_2O_2 groups (scale bars: 20 µm). *; P<0.05, ***; P<0.001 vs. the control group, and ^^; P<0.01 vs. the H_2O_2 group.

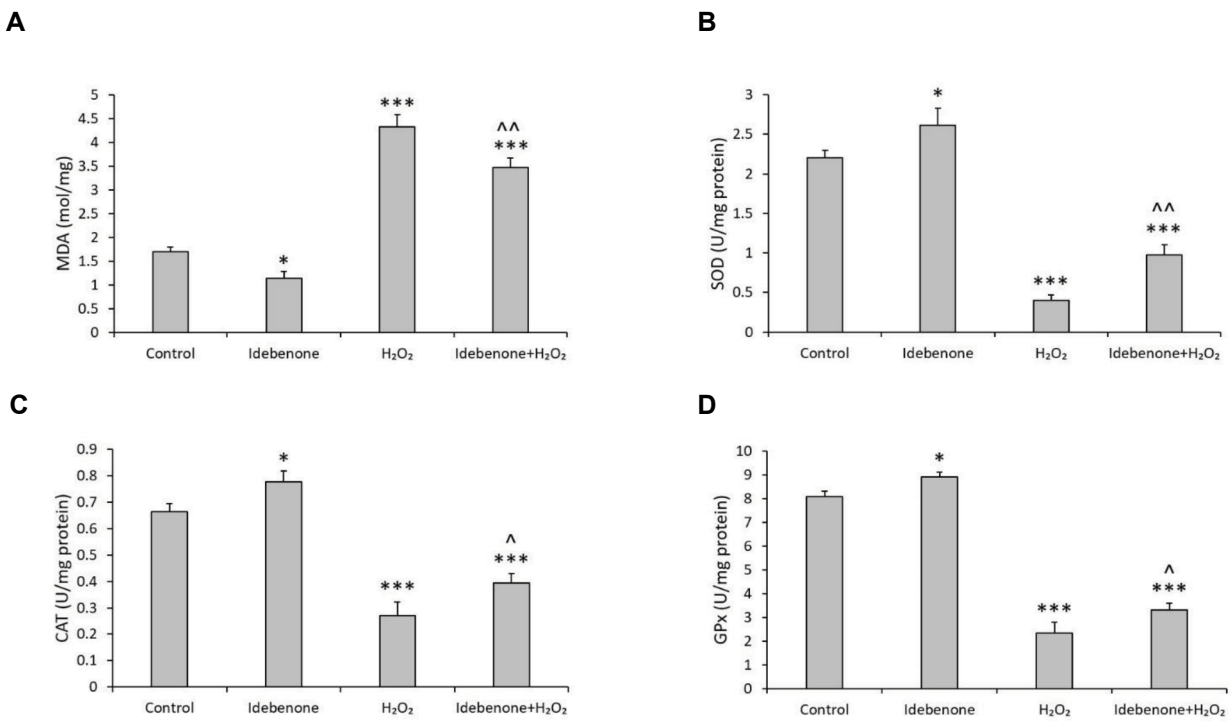

Fig.3: Effects of idebenone and H_2O_2 on oxidative stress biomarkers in Schwann cells. **A.** Effects of idebenone and H_2O_2 on malondialdehyde (MDA) level. **B.** Superoxide dismutase (SOD), **C.** Catalase (CAT), and **D.** Glutathione peroxidase (GPx) activity in Schwann cells. *; P<0.05, ***; P<0.001 vs. the control group, ^; P<0.05, and ^^; P<0.01 vs. the H_2O_2 group.

Western blot assay

MPZ, PMP22, Bax, and Bcl-2 protein expressions were assessed by Western blot. The results showed that the addition of H_2O_2 significantly stimulated Schwann cells and increased MPZ and PMP22 expressions compared to the control group (P<0.001). There was no significant difference between the idebenone group and the control group in terms of MPZ (P=0.297) and PMP22 (P=0.409) expression. Treatment with idebenone+H_2O_2 resulted in significantly greater MPZ (P=0.013) and PMP22 (P=0.006) expressions compared to the H_2O_2 group. Figure 5 presents the two-way ANOVA results for MPZ and PMP22 after idebenone treatment ($F_{(1,8)}$=18.5 and $F_{(1,8)}$=21, P=0.003 and P=0.002, respectively), H_2O_2 treatment ($F_{(1,8)}$=1303.2 and $F_{(1,8)}$=440.1, P<0.001 and P<0.001, respectively), and idebenone+H_2O_2 treatment ($F_{(1,8)}$=2.56 and $F_{(1,8)}$=5.1, P=0.15 and P=0.054, respectively).

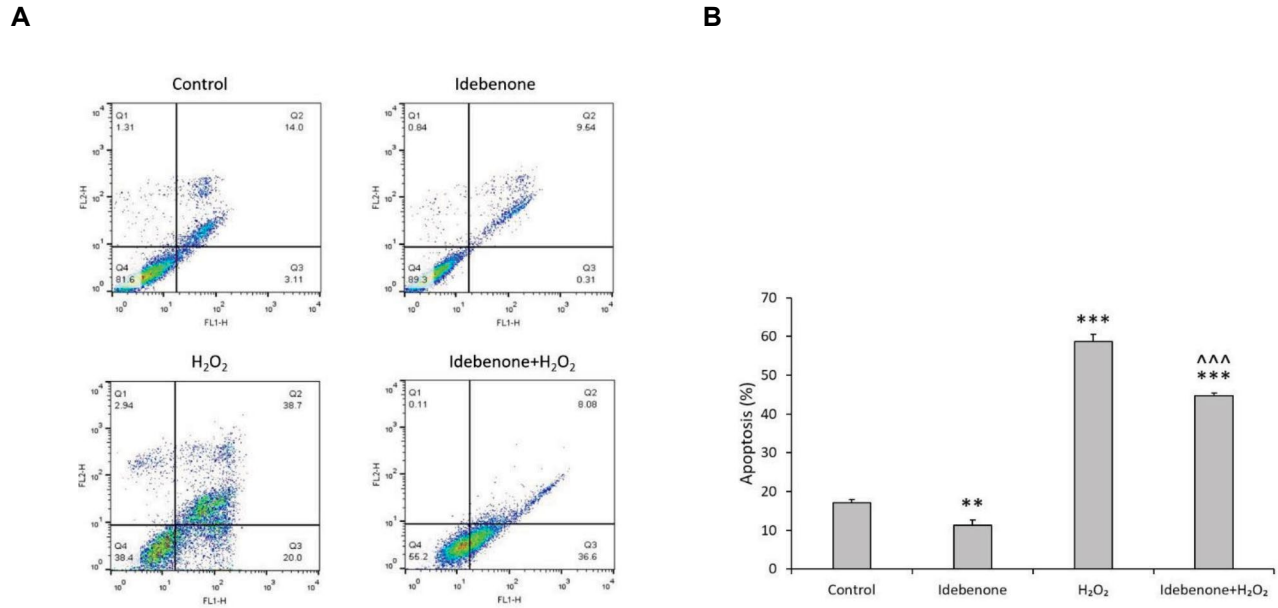

Fig.4: Effects of idebenone and H_2O_2 on the percentage of Schwann cell apoptosis by annexin V staining. **A.** The flow cytometry dot plots show that the apoptotic cells have a high-fluorescence intensity in quadrants 2 and 3. **B.** The percentages of apoptosis in the control, idebenone, H_2O_2, and idebenone+H_2O_2 groups. **; P<0.01, ***; P<0.001 vs. the control group, and ^^^; P<0.001 vs. the H_2O_2 group.

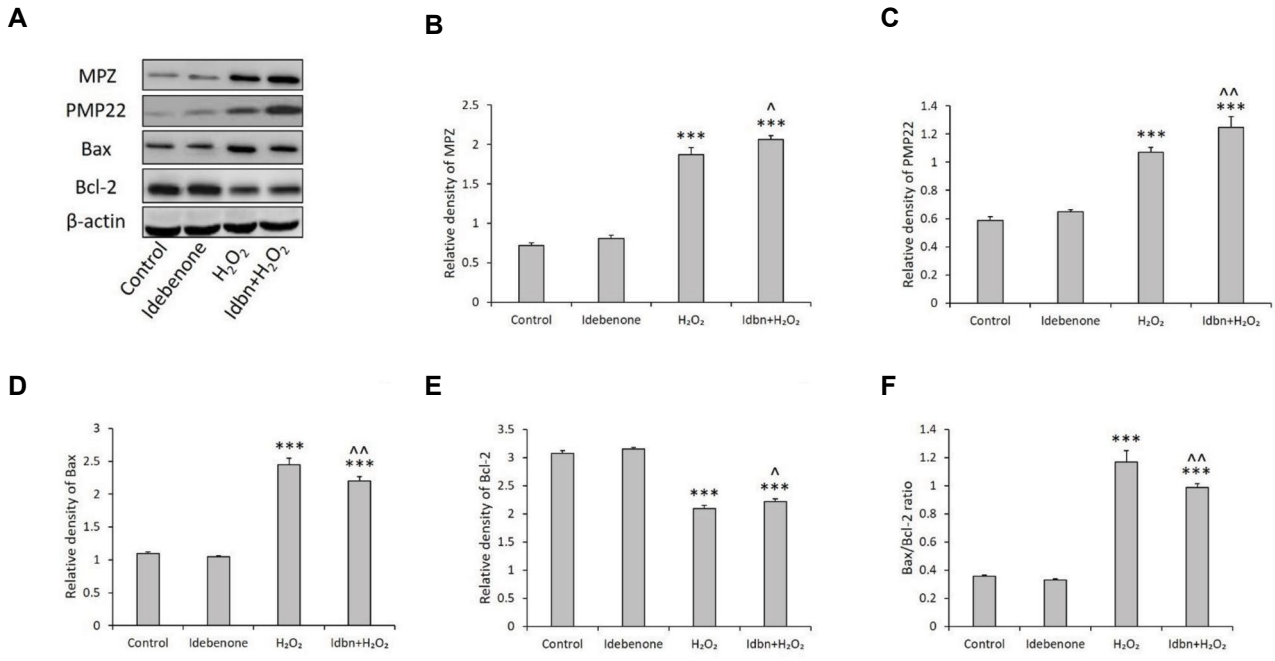

Fig.5: Effects of idebenone and H_2O_2 on the expressions of myelin protein zero (MPZ), peripheral myelin protein 22 (PMP22), Bax, and Bcl-2 proteins in Schwann cells by Western blot. **A.** Immunoblot of MPZ, PMP22, Bax, Bcl-2, and β-actin proteins. Expressions of **B.** MPZ, **C.** PMP22, **D.** Bax **E.** Bcl-2 proteins, and the **F.** Bax/Bcl-2 expression ratio. ***; P<0.001 vs. the control group, ^; P<0.05, and ^^; P<0.01 vs. the H_2O_2 group.

Assessment of Bax and Bcl-2 protein expressions showed that there were no significant differences between the idebenone group compared to the control group (P=0.745 and P=0.213, respectively). Bax protein expression in the idebenone+H_2O_2 group was significantly lower compared to the H_2O_2 group (P=0.007) and Bcl-2 protein expression in the idebenone+H_2O_2 group was significantly higher compared to the H_2O_2 group (P=0.026). The expression ratio for Bax/Bcl-2 significantly increased with the addition of H_2O_2 compared to the control group (P<0.001); there was no significant difference between the idebenone group compared to the control group (P=0.874). In the idebenone+H_2O_2 group, the expression ratio of Bax/Bcl-2 proteins was significantly lower compared to the H_2O_2 group (P=0.004). Figure 5 shows the results of two-way ANOVA for Bax, Bcl-2, and the Bax/Bcl-2 ratio after idebenone treatment ($F_{(1,8)}$=16.36, $F_{(1,8)}$=17 and $F_{(1,8)}$=17.85, P=0.004, P=0.003 and P=0.003, respectively), H_2O_2 treatment ($F_{(1,8)}$=1130.3, $F_{(1,8)}$=1457.9 and $F_{(1,8)}$=899.8, P<0.001, P<0.001 and P<0.001, respectively), and idebenone+H_2O_2 treatment ($F_{(1,8)}$=6.8, $F_{(1,8)}$=1.13 and $F_{(1,8)}$=10, P=0.031, P=0.32 and P=0.013, respectively).

Discussion

Schwann cells are the main cells for myelination and regeneration of peripheral nerves. Neuroprotective drugs may accelerate axonal regeneration following a peripheral nerve injury (18). Idebenone is a synthetic antioxidant that has powerful antioxidant properties. Idebenone is more effective than natural antioxidants such as coenzyme Q10, vitamin C, and vitamin E (11). In the present investigation, we explored the antioxidant effects of idebenone on viability, apoptosis, and functionality of Schwann cells under oxidative stress induced by H_2O_2 *in vitro*. The outcomes indicated that an optimal idebenone dosage effectively safeguarded Schwann cells during oxidative stress, and led to enhanced cellular function. These findings align with previous research that demonstrated the protective effects of idebenone on various cell types *in vitro*, including bone marrow mesenchymal stem cells, microglial cells, and brain cortical neurons (11, 19, 20).

In the current study, Schwann cells were cultured and assessed for S-100 protein expression, as a marker for Schwann cells. Next, the cells were exposed to idebenone and/or H_2O_2 to evaluate the viability, apoptosis, and function of these cells. H_2O_2 increased apoptosis and decreased both viability and function of the Schwann cells by increasing oxidative stress. In contrast, idebenone demonstrated a protective effect on Schwann cells by mitigating oxidative stress and enhancing cellular function. In similar studies, the effects of idebenone on retinal pigment epithelial and retinal ganglion cells exposed to H_2O_2 were investigated and the results showed that idebenone protects these cells from oxidative damage by regulating the mitochondrial pathway of apoptosis (14, 21, 22).

The level of the oxidant marker MDA and activities of SOD, CAT, and GPx, as antioxidant biomarkers, were measured to evaluate the antioxidant effects of idebenone on Schwann cells under oxidative stress induced by H_2O_2. Idebenone decreased the MDA level and increased the activity of antioxidant biomarkers in these cells. These results agreed with other studies. Lone et al (23). showed that idebenone improved the quality of ram sperm during cryopreservation by decreasing oxidative stress, including decreasing MDA levels and increasing SOD, CAT, and GPx activities. Nagy and Zs -Nagy (24) have demonstrated that idebenone increases the activity of endogenous enzyme antioxidants such as SOD, CAT, and GPx in the brains and livers of rats.

We used the MTT assay to evaluate the effects of idebenone on Schwann cell viability under oxidative stress induced by H_2O_2. Idebenone could increase cell viability by increasing the activity of antioxidant enzymes and decreasing MDA levels. These results supported findings from other studies. Zhang et al. (11) have reported that idebenone increases the viability of bone marrow mesenchymal stem cells *in vitro* by protecting cells. Arend et al. (14) have demonstrated that idebenone increases the survival of retinal pigment epithelium cells by decreasing intracellular ROS and apoptosis.

Next, we investigated the effects of idebenone on Schwann cell apoptosis under oxidative stress conditions caused by H_2O_2. The expression ratio of Bax/Bcl-2 was analysed using Western blot, and the percentage of apoptotic cells were calculated by annexin V staining and flow cytometry.

Idebenone has a protective *effect* against oxidative stress within the mitochondria (25) and the ratio of Bax/Bcl-2 protein expression shows the mitochondrial pathway of apoptosis (26). Therefore, we examined the anti-apoptotic effect of idebenone on Schwann cells under oxidative stress by evaluating the expression ratio of these proteins. The results of annexin V staining confirmed the Western blot results. Idebenone probably decreased apoptosis of Schwann cells against H_2O_2 by increasing the activity of the antioxidant enzymes and protected the mitochondria. These results agreed with findings from other studies. Clementi et al. (21) showed that idebenone decreased retinal pigment epithelial cell apoptosis exposed to H_2O_2 by regulating the intrinsic mitochondrial pathway of apoptosis. Kernt et al. (27) have reported that idebenone increases the viability of optic nerve astrocytes after H_2O_2 treatment *in vitro* by reducing intracellular ROS and preventing apoptosis in these cells by increasing Bcl-2 expression, as an anti-apoptotic protein, and decreasing Bax expression, as a pro-apoptotic protein.

We evaluated the effect of idebenone on Schwann cell function under oxidative stress conditions by performing Western blot analysis of the MPZ and PMP22 protein expressions. MPZ and PMP22 are specifically expressed by Schwann cells, and increased expression of these proteins is associated with improved myelin sheath

formation, which contributes to enhanced peripheral nerve repair (28). The production of MPZ and PMP22 is very low under normal conditions. Damage to peripheral nerves stimulate Schwann cells causing a sudden increase in expressions of these proteins (29). For this possible reason, there was no observed significant difference between the idebenone and control groups in the current study. The groups where Schwann cells were stimulated with H_2O_2 had significant increases in protein expression. This increase in expression was significantly higher in the idebenone+H_2O_2 group compared to the H_2O_2 group. Idebenone probably increased MPZ and PMP22 protein expression by decreasing oxidative stress, improving mitochondrial function, and increasing ATP production in the Schwann cells. There is no similar report on the effect of idebenone on Schwann cell proteins; however, Liu et al. showed that quercetin and cinnamaldehyde could promote MPZ and myelin basic protein (MBP) expression in Schwann cells exposed to high glucose *in vitro* by inhibiting the ERK signalling pathway (30). Caillaud et al. (29) have reported that curcumin could increase MPZ and PMP22 protein expressions by inhibiting oxidative stress and thus improve remyelination of damaged rat sciatic nerve.

Following peripheral nerve injury, transcription of nuclear factor erythroid 2 (Nrf2), an intrinsic antioxidant system, is transiently inactivated and impairs Schwann cell plasticity (26, 31). Therefore, administering extrinsic antioxidants may help regulate the intrinsic antioxidant system and ameliorate the function of Schwann cells to repair damaged peripheral nerves (26, 32).

Nrf2 is an important transcription factor that can activate antioxidant reactions and plays an essential role in inflammatory responses and tissue remodelling. In addition to Nrf2, Kelch-like ECH-associated protein 1 (*KEAP1*) and antioxidant response element (*ARE*) are also essential genes to protect cells. Therefore, the Nrf2/KEAP1/ARE signalling pathway is one of the main cellular defence mechanisms against oxidative stress (5). Idebenone upregulates the Nrf2/KEAP1/ARE pathway to protect cells by activating NAD(P)H quinone oxidoreductase 1 (NQO1) which is a part of the cellular physiological response to stress (10).

Idebenone protects neural and glial cells from oxidative stress damage by inhibiting lipid peroxidation in cells and mitochondrial membranes (33). Wang et al. (20) have shown that idebenone protects cortical neurons against amyloid-beta toxicity in vitro by preventing collapse of mitochondrial function.

There were some limitations in this study. The effect of idebenone on Schwann cells was only investigated in vitro. In addition, we did not investigate any inflammatory factors, nor were the Schwann cells assessed at different time points.

Conclusion

The results showed that an appropriate dose of idebenone, a synthetic antioxidant, may protect rat Schwann cells against the harmful effects of H_2O_2 *in vitro* and improve the survival and function of these cells by reducing oxidative stress and apoptosis.

Acknowledgment

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Authors' Contributions

S.Z.; Contributed to conception, Study design, and Obtained funding. P.H.; Contributed to the experimental work, Data collection, and Evaluation, Drafted the manuscript, and Performed statistical analyses. All authors edited and participated in the finalisation of this manuscript, and approved the final version of this manuscript for submission.

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Sperm Parameters and Chromatin Integrity in Men Suffering from Celiac Disease: Insights into Reproductive Health, Case-Control Study

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Abstract

Objective: Celiac disease is a common chronic inflammatory condition of the small intestine caused by permanent intolerance to gluten/gliadin. It has been demonstrated that oxidative stress is one of the mechanisms that is involved in gliadin toxicity, and there is a correlation between oxidative damage with this disease. Similarly, increased oxidative stress was repeatedly reported in infertile men which led to low-quality of sperm function. Therefore, we aimed to assess sperm parameters and chromatin status in men with Celiac disease.

Materials and Methods: In this case-control study, semen samples were collected from 11 fertile men without Celiac and 10 men with diagnostic Celiac disease. Basic semen analyses were performed according to the World Health Organization (WHO) 2010 protocol. The percentage of sperm with persistence histones, protamine deficiency, DNA fragmentation, malondialdehyde (MDA), and intracellular reactive oxygen species (ROS) were assessed using aniline blue, chromomycin A3, sperm chromatin structure assay, thiobarbituric acid reactive substances (TBARS) assay, and diacetyldichlorofluorescein staining, respectively.

Results: Unlike the sperm parameters, which did not show significant differences between men with Celiac disease and fertile individuals, sperm chromatin maturation (persistence histones and protamine deficiency) and sperm DNA damage in men with Celiac disease were significantly higher compared to fertile individuals ($P<0.05$). In addition, the percentage of sperm viability in these individuals was significantly lower than that in the fertile individuals ($P<0.05$). We did not observe any significant differences in sperm lipid peroxidation and intracellular ROS levels between the two study groups ($P>0.05$).

Conclusion: Celiac disease affects sperm chromatin maturation and DNA fragmentation, emphasizing its impact on reproductive health.

Keywords: Celiac, Chromatin, Oxidative Stress, Sperm parameters

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Introduction

Celiac disease (Celiac sprue) or a gluten-sensitive enteropathy, is characterized by an immune response to gluten (1). This protein is rich in proline and glutamine and is not completely disintegrate by the digestive system and can lead to the formation of gliadin peptides. Since gliadin peptides act as immune system stimulants in the small intestine, the level of inflammation and damage in the intestinal lining increases, and absorption of some nutrients through the small intestine decreases, therefore, individuals with Celiac disease face malabsorption (1, 2). In light of previous findings, environmental factors alone are not enough to cause Celiac disease, and the presence of genetic predisposition is also essential for the occurrence of this disease. In addition, among autoimmune disorders, an increased prevalence of Celiac has been reported in individuals with autoimmune diseases such as liver, thyroid, and type 1 diabetes mellitus (1).

The gliadin peptide becomes negatively charged by an

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enzyme called tissue transglutaminase (tTg), especially TG2 enzymes. When gliadin reaches the lamina propria, it is deamidated, and as an antigen, it can bind to receptors located on the surface of antigen-presenting cells, which leads to the production of antibodies and inflammatory factors by immune cells (2). These receptors are heterodimers encoded by the human leukocyte antigen (HLA) gene located on the short arm of chromosome 6 (3). Among HLA alleles, those that encode HLA-DQ2 and HLA-DQ8 proteins increase a person's genetic susceptibility to Celiac disease. HLA-DQ2 proteins have a greater affinity for binding to gliadin than HLA-DQ8 proteins, thus increasing the risk of developing Celiac disease (4-6).

The available research indicates that certain regions of the gliadin sequence play an important role in the disease progression of Celiac by applying cytotoxic or immunomodulatory activity, whereas other regions may boost oxidative stress and stimulate the release of pro-inflammatory cytokines (7). Therefore, in light of the evidence at hand, it is thought that in the etiology of Celiac disease, two key factors including heightened inflammation and the disproportion between oxidative stress and antioxidant defense play an important role (8-12). Prior investigations have highlighted that exposure of individuals with Celiac to gluten can stimulate intracellular oxidative imbalance which is characterized by increased levels of lipid peroxidation and an oxidized/reduced glutathione ratio, as well as decreased protein-bound sulfhydryl groups. Furthermore, individuals with Celiac disease demonstrated significantly higher expression of inducible nitric oxide (NO) synthase in the intestinal wall, resulting in significantly elevated levels of NO. High concentrations of NO metabolites have also been detected in the plasma and serum of untreated patients with Celiac (10).

In addition, increased levels of oxidative stress and its detrimental effects on sperm structure and function are the main cause of male infertility. Leukocytes and excess residual cytoplasm in sperm are the primary sources of reactive oxygen species (ROS) production (13). Therefore, oxidative stress can lead to peroxidative damage to the cell membrane, induce mitochondrial mutations, and cause breakage of DNA strands and chromatin cross-linking (13, 14). Earlier investigations have consistently found that increased sperm DNA fragmentation has been conclusively associated with a raised risk of miscarriage, poor embryonic quality, and implantation failure (15), as well as an increased childhood disease burden (16).

Taking this information into account, it appears that increased levels of oxidative stress are common to both Celiac disease and male infertility. This rise in levels may affect not just the quality of sperm function but also have repercussions on the health of subsequent generations. Therefore, the current study aimed to investigate sperm parameters and function in individuals with Celiac. If sperm function is affected in these patients, before deciding to have a child, it is better to evaluate the individual's fertility potential by performing a sperm analysis, and if necessary, through anti-oxidant therapy or the use of assisted reproductive techniques to minimize the unpleasant effects of the disease.

Material and Methods

Ethics and study design

This case-control study was approved by the Royan Institute's Ethics Committee (IR.ACECR.ROYAN.REC.1400.083). Semen samples were obtained from a total of 11 fertile men without Celiac, and 10 men diagnosed as Celiac after 3-4 days of sexual abstinence either by masturbation in the clinic. The semen samples of fertile men were collected from individuals who had two children and were referred to Isfahan Fertility and infertility Center for family balancing. In addition, fertile individuals did not have known pathological features such as varicocele, leukospermia, hormonal disorders and/or obstruction, presence of cryptorchidism, vasectomy, abnormal liver function, smoking, alcohol consumption, anatomical disorders, klinefelter syndrome.

For all participants, the study process was explained and if they agreed to participate in this project, they signed the consent form. Considering that the prevalence of Celiac disease is very low (1%), to collect Celiac semen samples, the Celiac Association of Iran was requested to contact individuals willing to participate in this study. During 18 months, 11 individuals accepted to participate in this study and provided semen samples for sperm analysis and assessment of sperm functional tests.

Confirmation of patients with Celiac disease

Celiac disease was diagnosed through serological tests for Celiac-related antibodies including immunoglobulin A (IgA) tTg test, IgA endomysial antibody (EMA) serology when IgA- tTg was weakly positive, and IgG-tTg test in subjects with serum IgA deficiency (17, 18). In seropositive individuals, the Celiac diagnosis was confirmed histologically by taking samples from the duodenal bulb and distal duodenum. The histological characteristics associated with Celiac included raised intraepithelial lymphocytes, crypt hyperplasia, and/or villous atrophy (19).

Patients with discordance between serology and histology results underwent testing for Celiac disease-related HLA DQ2 and DQ8 (20). Finally, according to the combination of clinical, analytical, and histological data, the diagnosis of Celiac disease was confirmed by a gastroenterology specialist.

Semen analysis

After the liquefaction of semen, basic analyses including sperm parameters (semen volume, sperm concentration, count, motility, morphology, viability) were carried out according to the WHO 2010 protocol (21). Firstly, we assessed sperm motility by computer-

assisted sperm analysis (CASA) using a LABOMED CxL light microscope. Therefore, we loaded 10 µl of semen into a preheated sperm counting chamber with a cover slide. At least, the status of 200 sperm in at least five fields was evaluated. Four types of sperm movement for each sample such as fast progressive, slow progressive, non-progressive, and immotile were assessed, and the results were reported as a percentage of "sperm motility" and "progressive sperm motility". Sperm concentration was evaluated by a counting chamber (sperm meter, sperm processor, Aurangabad, India) using a LABOMED CxL optical microscope (magnification: 20x). Sperm morphology was evaluated using papanicolaou staining with a trained technician. For each sample, two smears were prepared on a glass slide and fixed with a preserving solution. The slides were then subjected to Papanicolaou staining with hematoxylin, eosin, and orange G dyes, which selectively bind to different components of the sperm cells for improved visualization. After staining, the slides were rinsed, dehydrated with alcohol washes, and mounted with a coverslip using a protective medium. Microscopic examination at high magnification (×1000) allowed for the observation and assessment of abnormalities in the head, neck, and tail of at least 200 sperm. The results were expressed as the "percentage of abnormal sperm morphology". Sperm viability was assessed by eosin-nigrosine staining, and Hypo osmotic (HOS) test according to WHO 2010 protocol (21).

Sperm functional tests

Sperm chromatin maturation (persistence histones and protamine deficiency) were assessed by aniline blue test (Fisher chemical, USA, A967), and chromomycin A3 staining (Sigma, USA, C2659), respectively. Sperm DNA damage (sperm chromatin structure assay), sperm intracellular Diacetyldichlorofluorescein staining (DCF), and sperm malondialdehyde (MDA) (TBARS: thiobarbituric acid reactive substances) were also evaluated.

A. Persistence histones: Briefly, two smears were prepared by washed sperm, and then fixed with 3% glutaraldehyde. Next, slides were stained with 5% aqueous aniline blue in 4% acetic acid. After dehydration of the slides, they were mounted with xylol. At least between 100-200 sperm for each sample were randomly assessed, and sperm head with dark blue dye was considered as "sperm with persistence histones" (22).

B. Protamine deficiency: Briefly, two smears were prepared by washed sperm, and then fixed with Carnoy solution. Next, slides were stained with 200 µl of CMA3 solution (0.25 mg/ml) for 20 minutes. After washing slides with phosphate-buffered saline (PBS), at least between 100-200 sperm for each sample were randomly assessed using an epifluorescence microscope (Olympus, Japan) equipped with appropriate filters (460-470 nm) at 1000x magnification. Sperm with insufficient protamine content appear light yellow, while sperm with normal protamine content appear dark yellow (23).

C. DNA fragmentation: The SCSA® was carried out following the guidelines of its developer (24). After determining the sperm concentration, 2×10^6 sperm cells were mixed with TNE buffer (50 mM Tris HCl, pH=7.4, 100 mM NaCl, 0.1 mM EDTA, Merck, Darmstadt, Germany) to make a final volume of 1 ml. Then, a fifth of the spermatozoa suspension was subjected to sperm chromatin structure assay (SCSA) by adding 400 µl of acid-detergent solution followed by 1.2 ml acridine orange (AO) staining solution (Sigma, St. Louis, USA, A8097) for 30 seconds. The samples were analyzed using a FAX-Calibur cytometer (BD Biosciences, San Jose, CA, USA), counting at least 10,000 sperm cells. The results were reported conventionally, indicating the DNA fragmentation index % (DFI) and high DNA stainability % (HDS) scores.

D. Sperm intracellular ROS: Briefly, one million sperm per ml PBS was incubated with 0.5 µM DCFH-DA (Sigma Co, USA) at room temperature for 40 min, in a dark setting. Then, samples were analyzed using FACSCalibur flow cyt-ometer (Becton Dickinson, San Jose, CA, USA) with excitation wavelength 488 nm. For each sample, at a flow rate of <100 cells/s, 10,000 events were recorded in the forward light scatter/side light scatter (FSC/SSC) dot plot (25).

E. Sperm MDA: MDA is one of the products of lipid peroxidation. In this study, sperm MDA was assessed by using thiobarbituric acid reactive substances (TBARS) assay according to the method described by Esterbauer and Cheeseman (26). Briefly, $5\text{-}10\times10^6$ sperm cells were mixed with 400 µl of TBA reagent solution containing 10% trichloroacetic acid and 0.67% thiobarbituric acid. The mixture was then incubated for 45 minutes in a boiling water bath at 95°C. After cooling, the samples were centrifuged at 500 g for 6 minutes, and the resulting supernatant was subjected to spectrophotometric analysis at a wavelength of 532 nm using a spectrophotometer. A standard substance, 1,1,3,3-tetramethoxypropane, was used for comparison, and its absorbance was evaluated at 532 nm against a standard curve. MDA levels were quantified using a calibration curve ranging from 0.2 to 1.5 nmol, and the results were expressed as nmol/number of cells.

Statistical analysis

The statistical analyses were performed using IBM SPSS Statistics software, version 22 (IBM Corp., Chicago, IL, USA). To assess differences between the two groups, independent samples t tests were utilized. Except for four parameters (abnormal sperm morphology, %, intensity of DCF staining, and sperm DNA damage) which were not normally distributed and underwent transformation, all other data exhibited a normal distribution. Exact P values were reported in the results and figures. Descriptive statistics were presented as mean ± standard deviation (SD), and statistical significance was determined at a threshold of $P<0.05$.

Results

Comparison of sperm parameters between fertile and Celiac men

As illustrated in Figure 1, there were no significant differences (P>0.05) in sperm parameters, including semen volume (P=0.458), sperm concentration (P=0.466), sperm count (P=0.783), sperm motility (P=0.818), progressive motility (P=0.740), and abnormal sperm morphology (P=0.821), between men with Celiac disease and men with proven fertility. However, the percentage of sperm viability, as assessed by eosin-nigrosine staining (42.8 ± 15.56 vs. 59.7 ± 13.38, P=0.018), and the HOS test (60.09 ± 14.58 vs. 75 ± 14.58, P=0.025) were significantly lower in men with Celiac disease compared to fertile individuals.

Comparison of sperm chromatin between fertile and Celiac men disease

Sperm chromatin status was assessed using aniline blue staining to evaluate the persistence of histones, and chromomycin A3 staining for assay protamine deficiency in sperm. In addition, SCSA assay was employed to evaluate the percentage of sperm DNA damage, and the percentage of high DNA stainability. The results as depicted in Figure 2, revealed a significantly higher mean of persistence histones (70.30 ± 17.36 and 42.36 ± 14.44, P=0.001), protamine deficiency (63.95 ± 12.78 and 40.80 ± 15.27, P=0.002), and sperm DNA damage (35 ± 18.25 and 22 ± 4.41, P=0.044) in 2010 disease compared to fertile individuals, respectively.

Comparison of sperm lipid peroxidation, and intracellular reactive oxygen species between fertile and Celiac men

The oxidative stress status in sperm was assessed using TBARS (1.74 ± 0.84, and 2.22 ± 0.55, P=0.179), and DCFH-DA (14.2 ± 12.17 and 25.25 ± 19.96, P=0.197) assays to detect sperm lipid peroxidation specifically MDA, and intracellular ROS in sperm, respectively. Analysis of data presented in Figure 3 revealed no significant differences (P>0.05) in these markers between men with Celiac disease and fertile individuals.

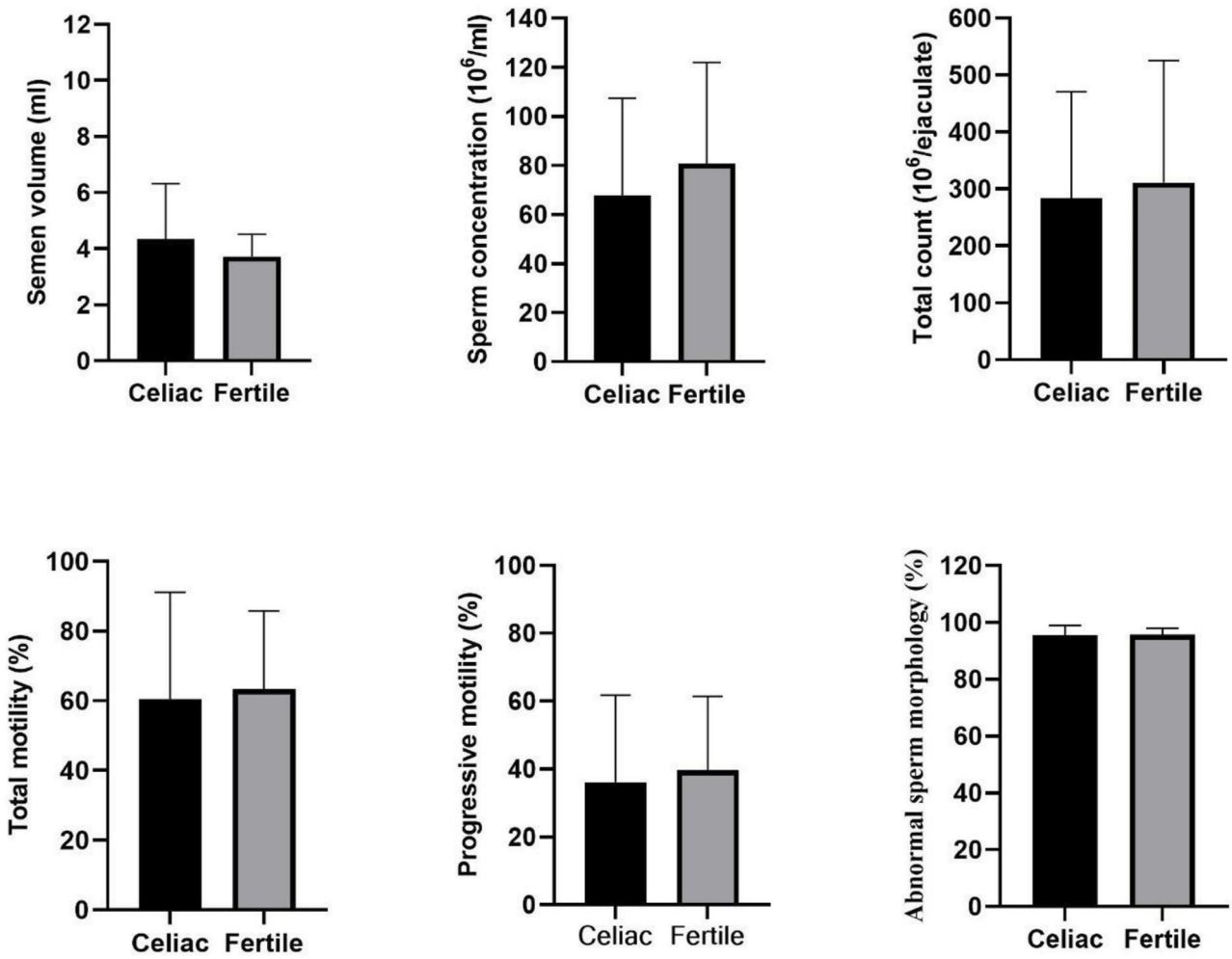

Fig.1: Comparison of semen parameters between 10 men with Celiac disease and 11 fertile men. Data are presented as mean ± standard deviation (SD).

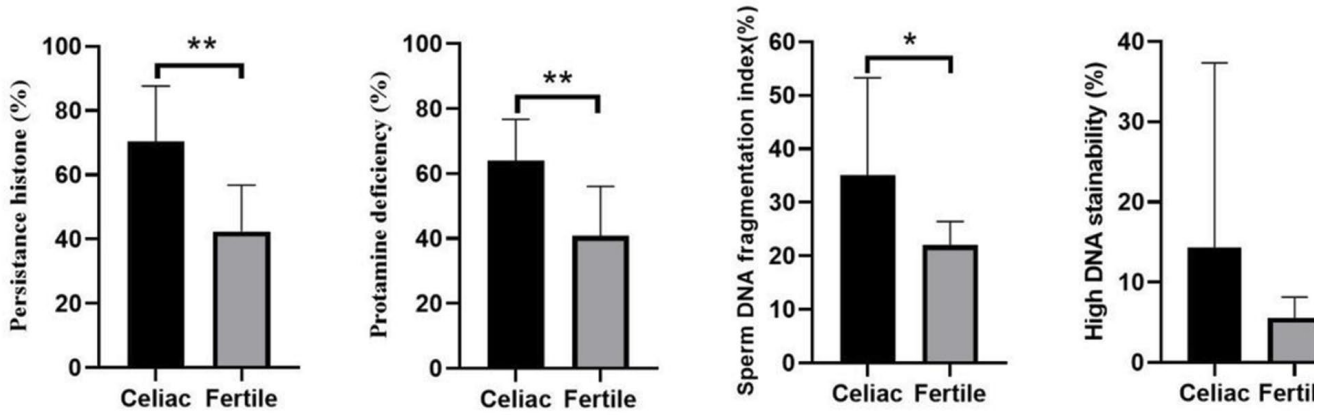

Fig.2: A comparison of sperm chromatin status between 10 men with Celiac disease and 11 fertile men. Sperm persistence histones were assessed by aniline blue, sperm protamine deficiency was assessed by chromomycin A3 staining, and DNA fragmentation was assessed by sperm chromatin structure assay (SCSA®). Statistical significance was set at P<0.05. Data are presented as mean ± standard deviation (SD). *; P<0.05 and **; P<0.01.

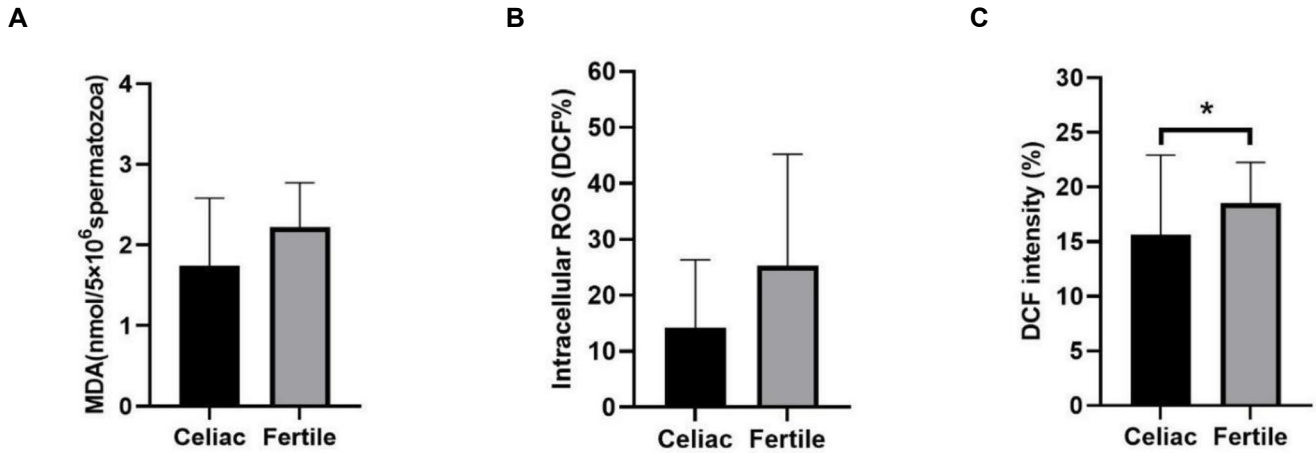

Fig.3: A comparison of oxidative stress status in sperm between 10 men with Celiac disease and 11 fertile individuals. MDA, and intracellular ROS in sperm were assessed using thiobarbituric acid, and DCFH-DA assays, respectively. Data are presented as mean ± standard deviation (SD). *; Statistical significance was set at P<0.05, MDA; Malondialdehyde, ROS; Reactive oxygen species, and DCF; Diacetyldichlorofluorescein.

Discussion

The activation of inflammatory pathways and the disproportion between the level of oxidative stress and antioxidant defense are extensively acknowledged as fundamental elements in the onset and progression of numerous diseases (27). The basic knowledge of these pathways to suppress possible damages at the cell and tissue level can be essential to make the accurate decision in treatment. The literature on this topic indicates that Celiac disease as one of these diseases associated with elevated levels of inflammation and oxidative stress (10). Therefore, this study sought to investigate whether this condition can impact sperm function in individuals diagnosed with Celiac disease.

The results of the current study clearly show that in Celiac patients, sperm parameters such as concentration, sperm count, motility, and morphology were not affected compared to the fertile group. In addition, there was no significant difference in the markers of oxidative stress level in the semen sample of these individuals compared to fertile men. But, the important point here is that sperm chromatin damage, including DNA integrity and sperm chromatin packaging, was significantly damaged in Celiac individuals compared to fertile men. Since half of the future genome of the fetus is provided by the father's genome, therefore, sperm chromatin damage can affect the fertility results of couples and even the health of the future generation (28, 29). Therefore, from our results, it seems that the process of sperm production and differentiation during the spermatogenesis, from the point of view of

sperm count and morphology, was normal in Celiac individuals, and even during the passage through the epididymis, as the mean percentage of sperm motility were similar between the two groups, but the packaging of sperm chromatin during spermiogenesis, which can be one of the main causes of DNA damage in the sperm sample was reduced in the Celiac group. Unlike our studies, Foss (30) have diagnosed the semen samples of two individuals affected by the Celiac disease as oligo-asthenozoospermia and reported that a gluten-free diet resulted in an improvement in both sperm concentration and motility.

Regarding the effect of Celiac disease on sperm parameters, recently, a review paper has been published in which the authors have shown that the number of articles in this direction, as well as the number of Celiac cases in each study, is very low (31). One of the limitations of our study was the low sample size of men with Celiac disease who were included in the study. However, one of the strengths of this study was the examination of sperm chromatin structure and DNA integrity. Although sample size in this study was low, the mean percentage of sperm DNA damage, protamine deficiency, and persistence histones in men with Celiac disease was significantly higher than in the control group. Considering the importance of the health of the father's genome as half of the genome of the future embryo, it is suggested that individuals with Celiac disease check the quality of sperm function before deciding to have children. Therefore, it is very difficult to judge the impact of this disease on the process of spermatogenesis and the fertility potential of men. In this regard, three old research papers have been published by Farthing group (32-34) regarding the effect of Celiac disease on sperm parameters. In 1982, these authors assessed sperm parameters in 28 men with Celiac and reported abnormalities in sperm motility, morphology, and 7% hypogonadism (31, 32). In 1983a, they demonstrated increased plasma testosterone and free testosterone index, reduced dihyvdrotestosterone, and raised serum luteinising hormone, a pattern of abnormalities indicative of androgen resistance in men with Celiac disease (33, 34). They also showed (1983b), sperm concentration was reduced (less than 40 million/ml) in 1 of 16 (6%) men with Celiac disease. Reduction of sperm motility (69% of Celiac) and increased proportions of abnormal spermatozoa (54% of Celiac) also occurred in these individuals (34).

The point that should be kept in mind is that the evaluation of sperm parameters in these very old studies was based on the thresholds defined by the first guidelines of the World Health Organization (1980), which present the normal range of sperm concentration between 20 and 200 million/ml, the percentage of sperm motility and normal morphology more than 60% and 80.5, respectively (35). Years later, Zugna et al. (36) only compared the number of children born from 7121 men with Celiac disease and 31,677 age-matched reference male controls that analysis of data showed 9,935 and 42,245, children in Celiac and control groups, respectively. Adjusting for age, calendar period, and parity and stratifying by education, the overall fertility hazard ratio in men with Celiac disease was 1.02 (95% confidence interval, 0.99-1.04). These authors reported normal fertility in men with Celiac disease. The point to keep in mind is that celiac disease, a multiorgan disorder, not only affects the gastrointestinal tract but also impacts endocrine organs involved in male reproductive health. Boys with celiac disease may experience androgen resistance, characterized by reduced serum dihydrotestosterone levels and elevated luteinizing hormone levels, leading to gonadal dysfunction. The exact mechanisms behind these reproductive disorders are not fully understood, but hypotheses propose a possible link to selective malabsorption of essential micronutrients (e.g., zinc, iron, folic acid, and fat-soluble vitamins) crucial for hormone metabolism, as well as autoimmune mechanisms. Hyperprolactinemia, commonly observed in individuals with celiac disease, may contribute to infertility by inhibiting the hypothalamus-pituitary axis and modulating the immune system. Timely diagnosis and management of celiac disease not only offer potential benefits for celiac disease recovery but also have the potential to improve an individual's fertility potential through the reversible nature of spermatogenesis. Further research is necessary to substantiate these findings (37, 38).

Conclusion

We found no significant differences in sperm parameters between Celiac and fertile individuals. However, we conducted a more comprehensive assessment by investigative sperm chromatin maturation and sperm DNA fragmentation in these individuals. We observed significant damage to sperm chromatin and increased sperm DNA fragmentation. This highlights the potential consequences of Celiac disease, which can impact various aspects of their health. Therefore, it is crucial to address concerns regarding the effects of Celiac disease on reproductive potential and the well-being of their children. To alleviate these concerns, the current best strategy is optimal supplementation with antioxidants, along with the use of assisted reproductive techniques if need. These approaches aim to minimize the unpleasant effects of the disease on the couple's fertility potential, as well as their emotional and mental well-being.

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Authors' Contributions

Sh.K.; Performed sperm parameters and Functional tests. M.T., M.H.N.-E.; Contributed to the conception, Design, Coordination of the study, Data analysis, and Revised the manuscript. M.H.E., F.M., N.J.; Provision of study patients, Diagnosis, Sample collecting, and Revision of the manuscript. All authors read and approved the final manuscript.

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2023, A Landmark Year in Biomedical Research; A Turning Point in Medical History

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Abstract

The rapid development of knowledge on healthy nutrition, and hygiene practices, as well as the advent of antibiotics and vaccines, has led to increased life expectancy in the recent century. The extended lifespan has brought new challenges for healthcare professionals, including the management of chronic degenerative diseases, malignancies, and autoimmune disorders. Advanced therapeutic medicinal products (ATMPs) have emerged as a promising frontier alongside conventional therapeutic modalities, offering innovative solutions through cell-based therapies, gene therapy, and tissue engineering. Recent years have witnessed remarkable advancements in regenerative medicine and the launching of innovative ATMPs. Numerous ATMPs have been registered and approved by regulatory agencies for the management of different diseases in 2023. The approval of groundbreaking therapies around the world has made 2023 an exceptional year. Novel ATMPs and the development of artificial intelligence (AI) in 2023 will pave the way for the integration of ATMPs and advanced technologies in personalized medicine, early diagnosis and targeted treatments.

Keywords: Cell-Based Therapies, Gene Therapy, Tissue Engineering

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Previously, health practitioners encountered significant challenges including infectious diseases, malnutrition, and poor hygiene while the development of science and widespread knowledge on healthy nutrition and hygiene, as well as the emergence of antibiotics and vaccines, have somewhat addressed these issues and led to an increase in life expectancy. However, the challenges facing healthcare professionals have changed with the increasing life expectancy, such as chronic diseases, malignancies, and autoimmune diseases (1, 2).

Encountering these clinical issues, advanced therapeutic medicinal products (ATMPs) emerged as a promising frontier alongside the conventional therapeutic methods. ATMPs encompass a diverse array of innovative approaches, including cell and cell-derivative-based therapies, gene therapy, and tissue engineering (3, 4).

In recent years, the field of ATMPs has seen significant advancements, with numerous innovative therapies being developed and tested. The year 2023 is known as a landmark year for ATMPs (5), with many groundbreaking therapies approved by the Food and Drug Administration (FDA), offering new hope to patients with complicated conditions. The approval of these therapies in 2023 is a testament to the rapid progress and potential of ATMPs in revolutionizing current medicine. Among numerous ATMPs for different disorders, 34 products have been approved by the FDA by the end of 2023. Seven ATMPs including CASGEVY™ [treatment of sickle cell disease (SCD)], ELEVIDYS™ [treatment of Duchenne muscular dystrophy (DMD) with a confirmed mutation in the DMD gene], LANTIDRA™ (donislecel) (treatment of type 1 diabetes), LYFGENIA™ (treatment of SCD), OMISIRGE® (in patients with hematologic malignancies who are planned for umbilical cord blood transplantation following myeloablative conditioning to reduce the time to neutrophil recovery and the incidence of infection), ROCTAVIAN™ (treatment of severe hemophilia A), VYJUVEK® (treatment of wounds in patients with dystrophic epidermolysis bullosa) were approved by FDA in 2023 (6). Among the mentioned products, CASGEVY and LYFGENIA™ are a pair of gene therapies for SCD which work through gene-editing and gene-addition approaches, respectively. CASGEVY™ and LYFGENIA™ are the first cell-based gene therapy products against SCD. Both therapeutic methods use autologous blood stem cells in 12 years and older patients with SCD and recurrent or history of vaso occlusive crises (VOCs) (7). CASGEVY™ was approved

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by the FDA in December 2023 and is known as the first gene editing therapeutic method in humans. This product which is based on CRISPER technology can be directed to cut DNA in targeted areas, enabling the ability to accurately remove, add or replace DNA where it was cut (8-10). Lyfgenia™, the other approved ATMP for SCD, is a revolutionary gene therapy that alters the patient's blood stem cells to generate a gene therapy-based corrected hemoglobin that mimics the function of a specific type of healthy adult hemoglobin unaffected by SCD.

The market authorization for ReColorCell®, the first approved ATMP in Iran, was approved in 2023 by the Iranian FDA (IR-FDA). ReColorCell® is a cell-based therapeutic product containing autologous melanocyte-keratinocyte for patients with vitiligo. This product is available in the form of a cellular suspension and is injected into the epidermal in spots resulting from vitiligo (11-13).

The development of ATMPs has the potential to provide targeted and personalized treatments that can address the underlying pathologies of diseases rather than just managing symptoms. This represents a significant shift towards more effective and sustainable healthcare solutions. In the future, ATMPs are expected to play a key role in the development of precision medicine, where treatments are tailored to individuals based on their genetic makeup and specific health needs. This personalized approach has the potential to improve patient outcomes alongside minimized side effects, reduce healthcare costs, and ultimately lead to a more efficient and effective healthcare system. Overall, the development and integration of ATMPs in medicine hold great promise for the future of healthcare and offer new perspectives on how we can approach and treat diseases. Furthermore, a combination of ATMPs and artificial intelligence (AI) algorithms holds great promise for early detection and treating a wide range of diseases by targeting specific molecular pathways. By utilizing AI and machine learning techniques, researchers can analyze vast amounts of data to identify potential drug targets, optimize vaccine design, and predict patient responses to treatment (14). The integration of AI in molecular targeting and vaccine development has the potential to revolutionize personalized medicine and enhance the efficiency of therapeutic interventions.

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Authors' Contributions

M.V.; Conceived the design of the study. B.S., M.V.; Reviewed literature and drafted the manuscript. All authors read and approved the final manuscript.

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In Vivo Vascularization of Endothelial Cells Derived from Bone Marrow Mesenchymal Stem Cells in SCID Mouse Model

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In this article published in Cell J, Vol 18, No 2, Jul-Sep (Summer) 2016, on pages 179-188, the authors found that Figure 2A was the same as the one that has already been published and it was confusing. The following figure's legend is corrected in reference 9.

The authors would like to apologies for any inconvenience caused.

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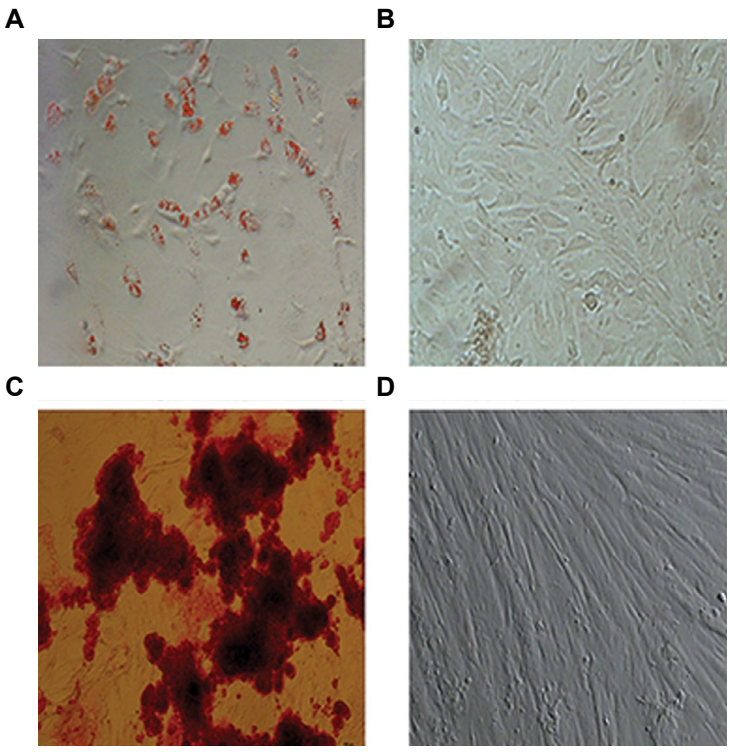

Fig.2: Characterization of mesenchymal stem cells (MSCs) by their ability to differentiate into adipocytes and osteocytes. **A.** The results of oil red-O staining in adipocytes that differentiated from MSCs (9), **B.** Negative control for adipocytes, **C.** The results of alkaline phosphatase (ALP) staining in osteocytes that differentiated from MSCs, and **D.** Negative control for osteocytes (magnification: ×10).

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