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Research Article

Introducing of a New Experimental Method in Semen Preparation: Supernatant Product of Adipose Tissue: Derived Mesenchymal Stem Cells (SPAS)

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Abstract

Human male infertility mostly is due to deficient semen parameters. One of the seminal plasma components providing chemical function of the ejaculate is growth factors functioning as paracrine, autocrine, and/or endocrine regulators of cell growth and differentiation. Adipose tissue-derived mesenchymal stem cells secret several proteins, such as cytokine, growth factors, and chemokine. On the other hand, due to contemporary semen preparation methods limitations, development of a new approach having least adverse effects and most efficiency is unavoidable. So, we decided to assess the effect of semen sample incubation with SPAS on semen parameters including motility, viability and DNA fragmentation.

Semen sample of infertile men attending infertility treatment center whose were 30-40 years old was incubated with supernatant product of adipose tissue- derived mesenchymal Stem cells (SPAS). Motility parameters, viability and DNA fragmentation were evaluated using CASA software, eosin- nigrosine staining and halosperm kit respectively. Then conventional method of semen preparation for Intra Uterine Insemination (IUI)- Density gradient centrifugation (DGC)- was compared with SPAS method.

Incubation of semen samples with SPAS (specially SP of 8×10^5 AD-MSCs for 40 minutes at 1:1 ratio**)** significantly increased motility parameters while decreased DNA fragmentation ($p\leq0.05$) and has no effect in terms of semen viability. Also, SPAS was significantly more influential in improvement of motility parameters than DGC ($p\leq0.05$).

Since effect of growth factors in improvement of motility and DNA fragmentation is documented in previous studies, it seems that SPAS can be an effective method which provides all these factors together.

Keywords: DNA fragmentation; **M**esenchymal stem cells; Motility parameters; Viability

Introduction

Inability to conceive after one year of unprotected intercourse is prevalent in approximately 15% of couples. In about 20% of infertile couples the male factor is merely responsible and in 30-40% of infertile couples it is a contributory factor [1]. Decreased numbers of spermatozoa, increased numbers of abnormal spermatozoa, poorly motile spermatozoa or a combination of these factors may lead to human male infertility [2]. An abnormal semen analysis is almost always a criterion for male infertility, although there are some other male factors which cannot be detectable in conventional semen analysis.

Semen consists of spermatozoa suspended in a fluid medium called seminal plasma. Seminal plasma is a complex fluid that mediates the chemical function of the ejaculate. One component of seminal plasma is growth factors which are polypeptides functioning as paracrine, autocrine, and/or endocrine regulators of cell growth and differentiation.

IGF-1 (Insulin-like growth factor-1) which has been suggested to have a direct or indirect role in spermatogenesis/steroidogenesis in the testes is one of these growth factors, so its derangement may be involved in male infertility [3,4]. Also it is known that HGF (hepatocyte growth factor) and its receptor are expressed in the mammalian male genital tract. In the genital tracts of mice expression of HGF is in a region-specific manner, with slight or no expression in testes and caput epididymis, and a strong expression in corpus and cauda epididymidis [5]. Moreover, in multiple tissues FGF (fibroblast growth factor) and FGFR (FGF receptor) expression has been reported [6,7] and both, ligands and receptors role in cell proliferation, differentiation, adhesion, survival, apoptosis, and motility have been implicated [8,9]. It is shown that percentage of both progressive and total motility of sperm will increase in exposure to FGF2. This effect was mediated by FGFR activation since sperm preincubation with the inhibitor prevented such increase [10].

Based on the ability of adult mesenchymal stem cells (MSCs) in self-renewal and multilineage differentiation, they are regarded as great candidates in the field of regenerative medicine [11]. These cells are mainly obtained from three sources: peripheral blood, bone marrow (BM) and adipose tissue (AT). However, the number of peripheral blood MSCs is few and not adequate. Due to ease of harvest and potential autologous application, AT and BM are the most widely used sources of MSC, especially in autologous cell-based therapies [12]. AT-MSCs had greater proliferative potential than BM-MSCs, while no significantly difference in colony efficiency was observed between the two types of cells. These cells secret several proteins, such as cytokine (interferon-γ), growth factors (bFGF, HGF, and IGF-1), and chemokine (stem cell-derived factor-1) [13].

So, based on the similarity between secreted proteins of MSCs and

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factors existing in seminal plasma as well as regarding their beneficial effect on sperm motility, we decided to use supernatant product of adult mesenchymal stem cells (SPAS) to see if it can improve the motility parameters of sperm or not.

Material and Methods

Experimental design

A total of 30 infertile men attending infertility treatment center of ACECR (academic center of education, culture and research, Qom branch) were assessed in this study. Patients provided informed consent to use the semen specimen that otherwise would have been discarded. The average range of participant age was 35 ± 5 years. The patient selection criteria were as following: 1) the count range should be ≥ 20×10^6/ ml; 2) motility should be between 20-50% without grade a.

All specimens were collected after a mandatory omission of sexual activity for 2-7 days. Semen specimens were placed into a 37°C incubator for 30 minutes to allow liquefaction to take place. After gentle vortexing, the samples were evaluated for volume and PH. Each specimen also was evaluated for sperm agglutination, motility, concentration and morphology by use of CASA software.

Then the specimens were incubated with supernatant product of adipose-derived mesenchymal stem cells (SPAS) of 5×10^5 and 8×10^5 cells (60 and 80 percent confluency of culture flask) at 1:1, 1:2 and 1:3 ratio while in every treatment sample the count of applied spermatozoa was constant (20×10^6/ml). Then all of these 6 treatment groups were placed in incubator. After 20 and 40 minutes the motility parameters were evaluated. The samples were then analyzed for sperm viability and DNA fragmentation.

In order to confirm the efficiency of our SPAS processing method with conventional procedure- density gradient centrifugation- a number of 15 infertile men whose semen was suitable candidate for IUI (Intrauterine insemination) were selected. Motility parameters were evaluated in each processing method and then compared statistically by use of SPSS software.

Sperm viability

In order to assess the effect of SPAS on semen viability, both treatment samples and control were evaluated after 40 min incubation using eosin- nigrosin staining.

0.67 g eosin Y (color index 45380) and 0.9 g of sodium chloride were dissolved in 100 ml distilled water. Then it was heated gently and 10.0 g nigrosin (color index 50420) was added to mixture. As soon as boiling was observed, the solution was removed from the hot plate and allowed to cool to room temperature. Then it was passed from a filter paper, sealed, and stored at 4°C in a dark glass bottle.

50 µl of each sample was mixed with equal volume of eosin-nigrosin solution. After 30 s, one drop of the mixture was transferred onto a microscope slide and smear was prepared. Then the slide was allowed to dry in air. Atleast 200 spermatozoa were evaluated under ×100 oil immersion with a bright field microscope. Live spermatozoa were left unstained (membrane intact) and dead spermatozoa stained pink or red (membrane damaged).

Assessment of motility

Sperm motility parameters were evaluated using CASA software. For each sample, at least 6 microscopic fields were analyzed and more than 300 motile sperm were evaluated. Motility parameters were measured and sperm motility was classified in Grade a (VAP ≥ 35 µm/s; STR ≥ 80%), Grade b (35 µm/s > VAP ≥ 10 µm/s), Grade c (10 µm/s > VAP ≥ 4 µm/s) or Grade d (VAP<4 µm/s). Percentages of total (Grade a+b+c) and progressive (Grade a+b) motility were recorded.

Assessment of DNA fragmentation

We used an improved sperm chromatin dispersion (SCD) test- the Halosperm kit (INDAS laboratories, Madrid, Spain)- to assess sperm DNA fragmentation. In brief, an aliquot of a semen sample was diluted to 10 million/mL in phosphate-buffered saline (PBS). Gelled aliquots of low-melting point agarose in eppendorf tubes were provided in the kit, each one to process a semen sample. Eppendorf tubes were placed in a water bath at 90-100°C for 5 minutes to fuse the agarose, and then in a water bath at 37°C. After 5 minutes of incubation for temperature equilibration at 37°C, 60 mL of the diluted semen sample were added to the eppendorf tube and mixed with the fused agarose. Of the semen–agarose mix, 20 µL were pipetted onto slides precoated with agarose provided in the kit, and covered with a 22- by 22-mm coverslip. The slides were placed on a cold plate in the refrigerator (4°C) for 5 minutes to allow the agarose to produce a microgel with the sperm cells embedded within. The coverslips were gently removed and the slides immediately immersed horizontally in an acid solution, previously prepared by mixing 80 µL of HCl from an eppendorf tube in the kit with 10 mL of distilled water and incubated for 7 minutes. The slides were horizontally immersed in 10 mL of the lysing solution for 25 minutes. After washing 5 minutes in a tray with abundant distilled water, the slides were dehydrated in increasing concentrations of ethanol (70%, 90%, 100%) for 2 minutes each and then air-dried.

For this study, a minimum of 500 spermatozoa per sample were scored under the _100 objective of the microscope. In this study semen samples including controls and the samples treated with SPAS of 8×10^6 MSCs at 1:1 ratio were analyzed after 40 minutes incubation.

Preparation of semen samples with density gradient centrifugation method

Density gradient media (Allgrad, Life global) was prepared in 40 and 80 % concentration by adding Hams F10 supplemented with HSA. The density gradient was prepared by layering the 80% medium under the 40% medium in a conical centrifuge tube. Using a sterile pipette, 2 ml of the liquefied semen sample was layered over the upper layer (40%) and centrifuged at 300 g for 15 minutes. After the centrifugation, most of the supernatant was gently removed and the pellet was placed into a new, clean tube. Then pellet is well resuspended in 5 ml of medium (Hams F10+2.5% HSA) to remove the density gradient medium. At the end of the centrifugation, the supernatant was removed and 5 ml of new medium was added. The centrifugation repeated again and the final pellet was resuspended in the sterile medium.

Isolation and culture of adipose tissues-derived mesenchymal stem cells

Adipose tissues were harvested from patients undergoing elective liposuction surgeries. Briefly adipose tissues were rinsed with phosphate buffered saline containing 100 U/mL penicillin (Gibco), and 100 mg/mL streptomycin (Gibco) and digested with collagenase I (Sigma), then adipose tissue-derived mesenchymal stem cells cultured in DMEM medium (DMEM, Sigma) supplemented with 10% FBS (Gibco), 100 U/mL penicillin (Gibco), and 100 mg/mL streptomycin (Gibco) at 37°C under 5% Co_2 and 95% humidity. The medium was changed every 4 days. When the number of cultured cells reached to 5×10^5 and 8×10^5, SPAS were collected and stored in -20°C.

Before using supernatant product (SP) of 5×10^5 and 8×10^5 adipose-

derived mesenchymal stem cells, their PH was assessed by PH meter and was as following: 7.1 and 7.6 respectively.

Microbial culture of SPAS

In order to be sure about lack of any bacterial contamination in SPAS, before use, we cultured SPASA in blood culture medium to provide a nutritious environment for bacterial growth. After 48 hours, we transferred it onto EMB and blood agar. No growth was observed after 24 and 48 hours of culture.

Statistical analysis

The statistical significances of the effects of SPAS on motility parameters and viability were determined using SPSS software by ANOVA test while paired T-test was used to analyze data of DNA Fragmentation. P-values ≤0.05 were considered to be significantly different.

Results

Motility parameters

Incubation of semen specimens with SPAS resulted in improved total motility or at least motility parameters including a, b, and progressive grade.

As it is displayed in Figure 1, 20 min incubation of semen samples with supernatant product (SP) of 5×10^5 adipose-derived stem cells (AT-MSCs) increased total motility just in 1:1 ratio group while at 1:3 ratio the number of grade d significantly decreased and added to grade a and b. So, progressive motility showed a significant increase in comparison to control group ($p \leq 0.05$). Moreover, grade a at 1:2 ratio increased more than the other treatment groups and as a result improved progressive motility in this group ($p \leq 0.05$).

After 40 min incubation of semen samples with supernatant product of 5×10^5 AT-MSCs, total motility of 1:1 ratio group, due to increase in grade a and b and decrease in grade d, significantly improved ($p \leq 0.05$). Also, in 1:2 and 1:3 ratio groups the percent of spermatozoa with grade c motility significantly decreased and added to grade a ($p \leq 0.05$). As a result in all the treatment groups we observed enhanced progressive motility (Figure 2).

Incubation of semen samples with supernatant product of 8×10^5 AT-MSCs for 20 minutes in all treatment groups improved percent of progressive and a grades significantly while just in 1:1 and 1:2 ratio groups grade b and c motilities showed significant increase and decrease respectively. The percent of grade d in 1:1 and 1:3 ratio groups were diminished significantly and just in 1:1 ratio group improvement of total motility was significant ($p \leq 0.05$) (Figure 3).

40 min incubation of semen samples with supernatant product of 8×10^5 AT-MSCs in all treatment groups caused to significant increase in a, b and progressive grades in price of significant decrease in grades c and d (decrease of grade d in 1:2 ratio group was not significant). The only significant increase in total motility belonged to 1:1 ratio group ($p \leq 0.05$) (Figure 4).

When we compared the effect of applied ratio in improving motility parameters, we found that totally 1:1 ratio was more influential than the other ratios. As it is shown in Figures 1-4, except the group which was treated for 20 minutes with SP of 5×10^5 AT-MSCs, in every other concentrations and incubation times we observed significant increase in total, a, b and progressive motility in price of decrease in grade d while percentage of grade c in groups treating with SP of 8×10^5 AT-MSCs for 20 and 40 minutes significantly decreased ($p \leq 0.05$).

DNA fragmentation

Using halosperm kit, the effect of processing of semen samples with SPAS was evaluated and as it is shown in Figure 5, after 40 minutes incubation with SP of 8×10^5 AT-MSCs, the percentage of spematozoa with fragmented DNA significantly decreased in comparison to control group.

Viability

In order to understand if the processing of semen with SPAS affect sperm viability adversely or not, all the treatment groups were assessed using eosin-nigrosine staining.

The obtained data showed no significant decrease in percentage of treatment groups in comparison with control (Figure 6).

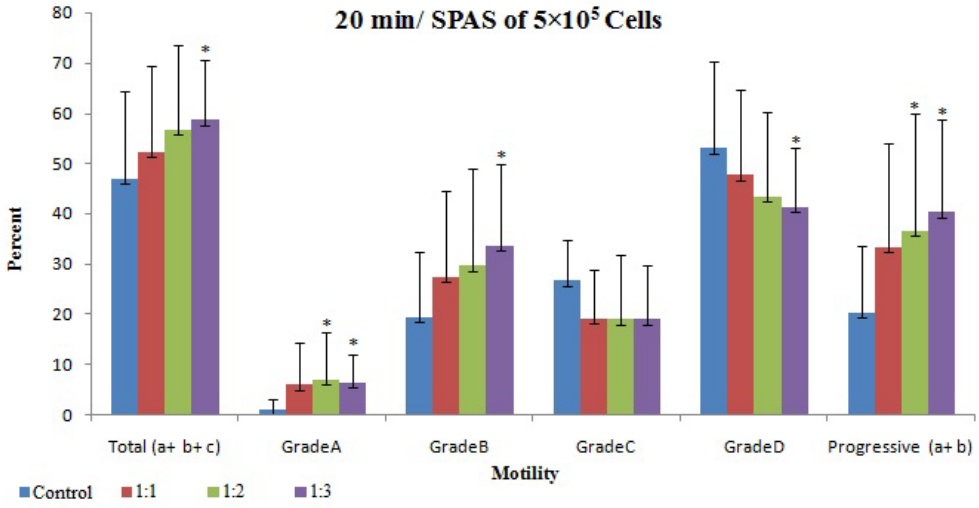

Figure 1: The comparison of motility parameters between control and treatment group after 20 min incubation with SP of 5×10^5 AT-MSCs (Error bars show standard deviation).

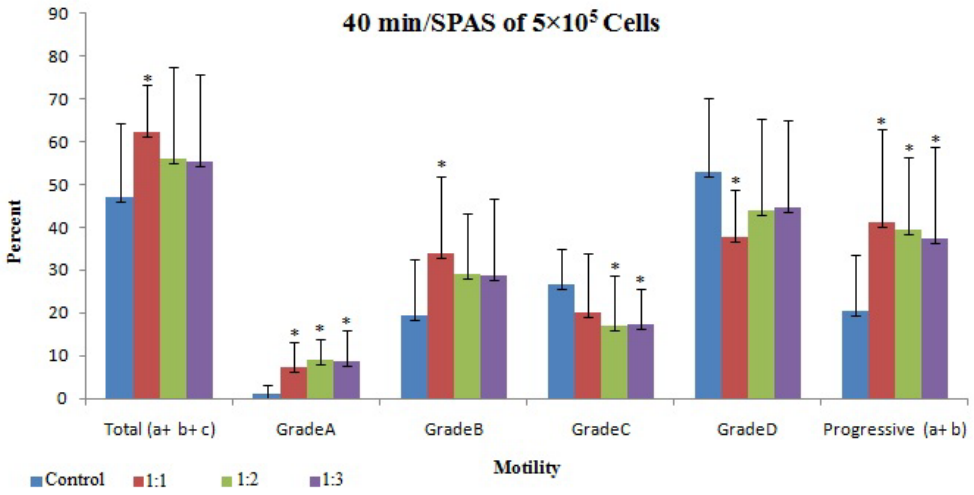

Figure 2: The comparison of motility parameters between control and treatment group after 40 min incubation with SP of 5×10^5 AT-MSCs (Error bars show standard deviation).

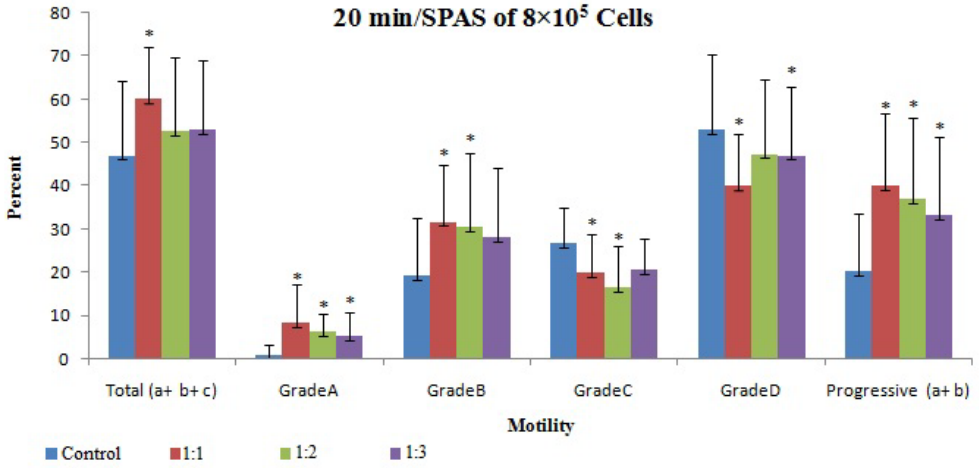

Figure 3: The comparison of motility parameters between control and treatment group after 20 min incubation with SP of 8×10^5 AT-MSCs (Error bars show standard deviation).

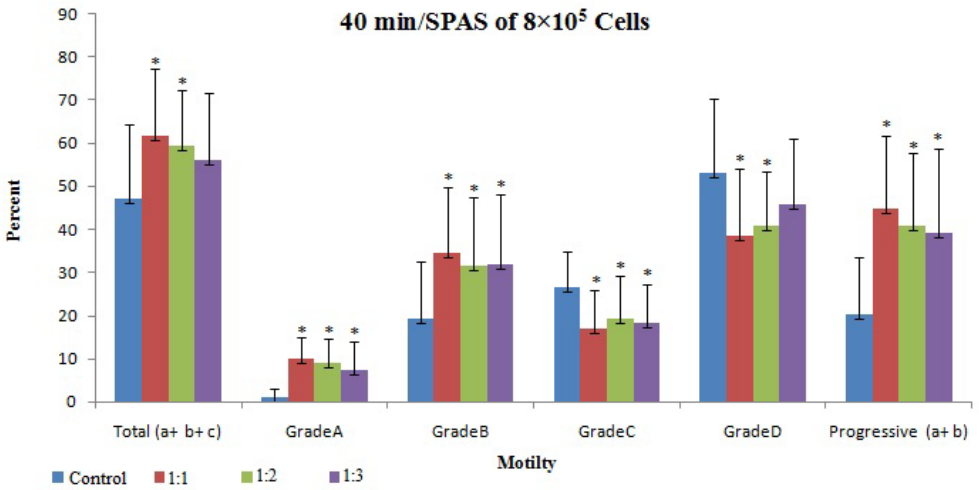

Figure 4: The comparison of motility parameters between control and treatment group after 40 min incubation with SP of 8×10^5 AT-MSCs (Error bars show standard deviation).

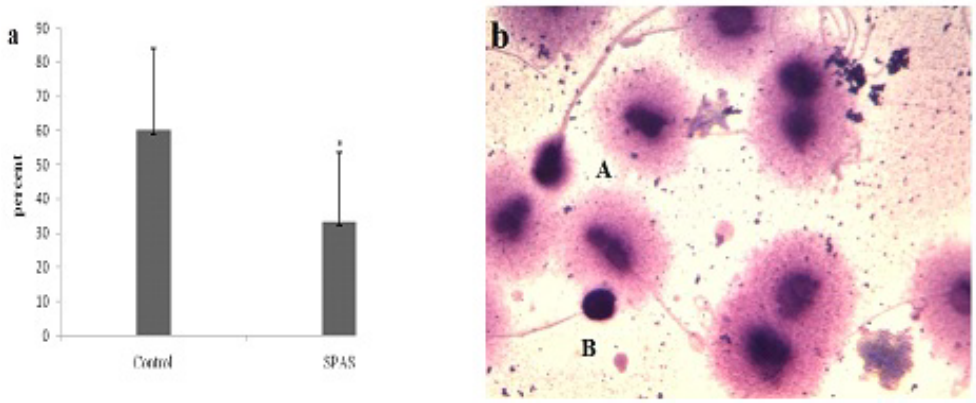

Figure 5: a) Assessment of DNA fragmentation in SPAS treated group and control. b) Human spermatozoa staining by halosperm kit. Normal sperm has big or medium size halo (A) while DNA fragmented sperm has small or no halo (B). *P*-values <0.05 were considered significant.*: Significant.

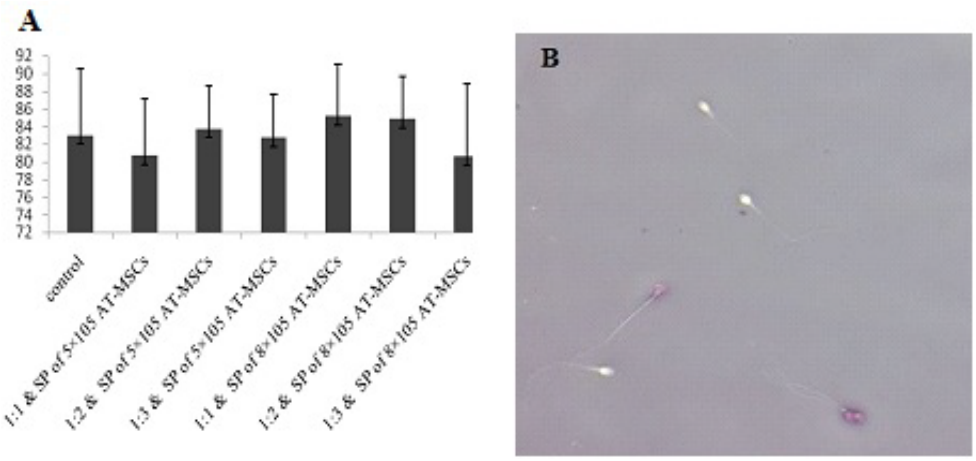

Figure 6: (A) The comparison of viability between different treatment groups and control after 40 minutes incubation. (B) Human spermatozoa staining with eosin-nigrosin dye assessed by oil immersion light microscopy at ×1000 magnification. Live spermatozoa appeared white whilst dead spermatozoa with disrupted membranes have taken up the eosin stain and appeared pink.

Groups \ Parameters	Total motility (a+b+c)	Grade a	Grade b	Grade c	Grade d	Progressive motility (a+b)
Control	61.67 ± 9.832^{a}	2.5 ± 2.739^{a}	30.83 ± 5.845^{a}	28.33 ± 6.831^{a}	38.33 ±9.832^{a}	33.33 ± 4.082^{a}
DGC	72.5 ± 13.323^{ab}	10.83 ±4.916^{b}	41.67 ±17.51^{ab}	20 ± 15.166^{ab}	27.5±13.323^{ab}	52.5 ± 20.187^{b}
SPAS	76.67 ± 9.832^{b}	15.83 ±5.845^{b}	46.67 ± 9.309^{b}	14.17 ± 8.612^{b}	23.33 ± 9.832^{b}	62.5 ± 12.942^{b}

Table 1: Comparison of motility parameters in control and treatment groups after processing with SPAS and DGC method.

SPAS vs. density gradient centrifugation method

Comparison of motility parameters in SPAS with DGC method showed a significant higher percent in terms of total motility, grades b, c and d between control and SPAS group while difference in these parameters was not significant between DGC and control group ($p \leq 0.05$). Although grade a and progressive motility of both DGC and SPAS in comparison to control group were significantly higher but in SPAS this difference was more than DGC group (Table 1).

Discussion

The accurate selection of the most efficient method for semen preparation before the assisted reproduction techniques such as IUI or intracytoplasmic sperm injection (ICSI)- which is strictly depends on the quality of the sample- is so critical [14]. A sample with appropriate count, motility and morphology of sperms usually is chosen for sperm washing or swim up method. By contrast, with a suboptimal quality sample, commonly density gradient centrifugation (DGC) is preferred. With the first methods, good quality sperms are obtained; while DGC is usually preferred for the greater number of motile spermatozoa selected from poor characteristics samples (low number, motility and morphology samples) [15].

However, there are some limitations in semen criteria to be selected for processing while severe oligoasthenozoospermia patients are not good candidate to undergo IUI due to low efficiency of semen preparation method. On the other hand, DGC produces Reactive Oxygen Species (ROS) and as a result despite recovering spermatozoa with improved motility, lowers DNA integrity [16,17]. So the requirement of a new method with least adverse effect on semen and maximum range of sample acceptance is unavoidable.

In this study the obtained result of semen sample incubation with SP of AT-MSCs (particularly with SP of 8×10^5 AT-MSCs for 40 minutes) not only caused to improve motility parameters, but also surprisingly decreased sperm DNA fragmentation. This observation can be due to the effective content of SPAS. It has been demonstrated that human MSCs secreted variety of growth factors, such as IL-6, VEGF, IL-8, G-CSF, SCF, IGF, HGF, IL-15, IL-10, NGF, PDGF-bb and bFGF [18-20]. Among these factors FGF, HGF, NGF and IGF-1 are known to affect semen parameters.

Selvaraju in 2012 has reported that variations in IGF-I levels in the seminal plasma can influence development, maturation and motility of the spermatozoa [21]. Regarding the presence of large amounts of HGF in the distal part of the epididymis and the acquisition of motility by immotile spermatozoa cultured with HGF, suggests that this growth factor can induce sperm motility [5]. In addition, it is demonstrate that FGFR1, 2, 3 and 4 are expressed in the human testis and sperm and their localization is limited to acrosomal region and flagellum. Since exposure to FGF2 leads to their phosphorylation and the activation of intracellular pathways, sperm FGFRs are functional. FGF2 has been localized in the female reproductive tract, secreted by the oviduct epithelial cells, the oocyte and the cumulus oophorus cells [22,23]. Due to findings that showed several kinases of the FGF/FGFR signaling pathways (in particular ERK and PI3K/Akt) have been involved in the maintenance of sperm motility, capacitation, acrosomal exocytosis and survival [24,25], it was proposed that FGF2 present in the endometrium, in the oviduct and in the oocyte vicinity could bind to sperm FGFRs and regulate those fertilization-related events [10]. In addition, incubation with FGF2 caused an increase in the percentage of motile cells and enhances sperm kinematics, suggesting that this system is involved in the regulation of sperm motility [10].

So we assume that incubation of semen sample with SPAS actually provides all of these factors together and induces motility parameters.

Assessing DNA fragmentation of semen samples after their incubation with SPAS showed a significant decrease. In comparison to control group (60 ± 24.01), SPAS method significantly decreased (33.2 ± 20.511) DNA fragmentation ($p \leq 0.05$). We suggest that decrease in DNA fragmentation is induced by the presence of growth factors such as IGF, bFGF and NGF.

NGF, discovered by Rita Levi-Montalcini, is a member of the neurotrophin proteins family (NT) and probably the most extensively studied member of NTs, possesses the ability to stimulate growth, differentiation and survival of neurons during development and after damage [26]. Many studies have shown that NGF not only is present in all stages of germinal cells from primary spermatocytes to mature spermatozoids, but also is detected in the Leydig cells and may play an important role in the semen parameters such as viability and motility of spermatozoa and DNA integrity [27,28].

In a study on rat ischemic brain hemisphere to evaluate the effect of bFGF on DNA fragmentation using terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick end labeling (TUNEL) histochemical methods combined with morphological criteria, it has shown that bFGF decreases DNA fragmentation through preventing reduction of immunoreactivity of the anti-apoptotic protein Bcl-2 in the ischemic hemisphere [29].

In order to explaining the role of IGF in decreasing DNA fragmentation, it have been reported that IGF as a survival growth factor induces Id2 expression in murine hematopoietic cells [30]. It is known that Id2 over expression in different cell types can promote cell viability and proliferation. This action is done via blocking the TGF-β-induced apoptosis [31].

Since one of the causes of double strand breaks in DNA is abortive apoptosis, it can be suggested that due to mentioned growth factors content of SPAS the rate of DNA fragmentation is decreased in SPAS treated group. So, having no significant effect on semen viability by use of SPAS can be justified too.

On the other hand, DNA repair does occur in developing sperm but it is terminated as transcription and translation stops post-spermiogenesis [32]. As a result, sperm have no mechanism to repair DNA damage that occurs during their transit and storage in the epididymis or post-ejaculation [32]. So it will be very problematic to refer DNA fragmentation improvement to transcriptional and translational mechanisms. As a result more study and focus on this issue is needed.

Comparing SPAS and conventional semen processing for IUI (DGC) showed that the ability of SPAS in improving motility parameters is more than DGC. Despite the ability of DGC in separating dead sperms, leukocytes and the other components of the seminal plasma, the type of motility do not change in applying this method. Whereas in SPAS method motility grade of sperm is improved to achieve higher progressive motility percentage as a key semen criteria for successful IUI.

Moreover, SPAS method can be use for every semen sample regardless of sperm count while this is a limiting factor in choosing DGC method. Also in DGC method chemical media such as allgrad, percoll, pure sperm and silicon are used which have toxic effects on semen specimen. In contrast, use of natural medium in SPAS method- which is more cost effective- induces less adverse effect on semen parameters. Furthermore in the process of DGC which is dependent on duration and centrifugation force, ROS is produced and exposure to high concentration of ROS can lead to disruption of mitochondrial and plasma membranes, which results in chromosomal and DNA fragmentation and causes a reduction in sperm motility [33,34]. After all, working with SPAS is easier technically.

Interestingly, growth factors, cytokines and chemokines content of SPAS have shown to have an important role in successful embryonic implantation [35]. So, it seems that use of SPAS can be beneficial for successful implantation. Of course for clinical application of SPAS and to assay its effect on fertilization rate, we are working on other Supplementary studies.

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