Correlation Between Protamine-2 and miRNA-122 in Sperm from Heroin-addicted Men: A Case-Control Study

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Purpose: Recreational use of illicit drugs is one of the main factors affecting male fertility. However, the mechanisms of heroin smoke-associated damage to mature spermatozoa are still completely unknown. The aim of this study was to concomitantly examine the levels of protamine-2 gene and protein concentrations, the amount of miRNA-122 in seminal plasma and semen analysis findings in heroin-addicted men.

Materials and Methods: In a case control study, twenty-four fertile men that lacked any recreational drug abuse were considered as the healthy group, and 24 addicted men who used only heroin for at least four months were selected as the addicted group. Semen samples were gathered by masturbation after 2 - 5 days of sexual abstinence. Following the preparation of a semen analysis by computer-assisted sperm analysis according to WHO (2010), the level of protamine-2 gene expression in sperm and miRNA-122 in seminal plasma was measured using real-time sqPCR. Also, protamine-2 protein concentrations were quantified by nuclear protein extraction, SDS-Page and western blotting.

Results: Among the studied variables, body mass index (27.75 ± 0.88 vs. 22.30 ± 0.36, $p = 0.001$), seminal pH (7.79 ± 0.06 vs. 7.58 ± 0.06, $p = 0.003$), white blood cell count in semen (1.69 ± 0.41 vs. 8.61 ± 1.73, $p = 0.001$), motility (65.51 ± 2.57 vs. 41.96 ± 3.58, $p = 0.001$) and survival rate (87.41 ± 1.00 vs. 71.50 ± 4.59, $p = 0.002$) of sperm cells was significantly different between the healthy and addicted groups. In addition, the levels of protamine-2 gene and protein expression in the addicted group (0.05 ± 0.02 and 0.10 ± 0.02, respectively) were significantly lower than the healthy group (3.59 ± 0.94 and 0.27 ± 0.06, respectively) ($p = 0.002$ and $p = 0.017$, respectively). Seminal miRNA-122 levels in addicted men (3.51 ± 0.73) were statistically higher than in healthy men (1.52 ± 0.54) ($p = 0.034$).

Conclusion: This is one study on human infertility that evaluates the effects of heroin on protamine deficiency and seminal small RNAs expression levels. Heroin abuse may lead to male infertility by causing leukocytospermia, asthenozoospermia, protamine deficiency, and seminal plasma miRNA profile alteration.

Keywords: protamine-2; miRNA-122; sperm; male infertility; heroin; illicit drugs; addiction

INTRODUCTION

Infertility is one of the major medical challenges that affects over 15% of couples worldwide. Approximately 50% of the infertile cases are due to male factors[(1)].

Semen abnormalities are one of the most common types of infertility due to multiple potential causes including inheritance, hormonal defects, drug abuse, (especially illicit drugs), and infection[(2,3)], but alarmingly, 60 to 75% of causes are still unknown or idiopathic[(4,5)]. It has been explored that drug abuse affects the hormonal balance and quality of semen, which leads to increased DNA fragmentation in sperm cells[(6)].

Although close scrutiny of the patient's history along with semen analysis to diagnose idiopathic infertility is necessary, it is not enough[(7,8)]. In recent years, alongside with conventional sperm parameters, the attention to molecular details, especially the seminal RNA content, has significantly grown.

Micro RNAs (miRNA) are a class of non-coding small RNAs which exist in serum and plasma, semen and other body fluids. However, their production pat-

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Received November 2019 & Accepted June 2020

Table 1. Gene primers.

Gene	Forward Primer	Reverse Primer
PRM-2	5'-CACGCACGAGGTGTACAGG-3'	5'-CAGTGTCTGCGCCTAAAGTGA-3'
B-actin	5′-TCCCTGGAGAAGAGCTACG-3′	5′-GTAGTTTCGTGGATGCCACA-3′
miRNA-122	5′- TGGAGTGTGACAATGG-3′	

terns differ in different disease[9]. The seminal plasma miRNAs are implicated in the regulation of spermatogenesis and gene expression in spermatozoa and the zygote during fertilization. These molecules were used as a novel, non-invasive biomarkers for the diagnosis of infertility and the classification of the types of them[10-12]. miRNA-122 is one of the high-expressing seminal miRNAs which alters in patients with azoospermia and asthenozoospermia[10,13]. However, the role of miRNAs, and more specifically, miRNA-122 in infertility are not yet clear[14,15]. Likely, recognizing this role will make miRNAs more reliable indicators for identifying and treating infertility.

Recognizing the role of miRNAs in semen comes down to this: miRNAs are bound to be used as more reliable biomarkers for diagnosing different types of infertility, and subsequently dictating their treatment protocol [14]. The health of the head and more specifically the nucleus of the sperm depends on the correct expression of the proteins and protamine genes. One of the issues raised in idiopathic infertility is protamination abnormalities[16]. In human, there are two types of protamine: protamine-1 (P1) and protamine-2 (P2) and they are expressed equally in the sperm cells[17]. The protamine mRNA (PRM) is expressed in round spermatids, whereas the translation is postponed until spermatid elongation[18,19]. According to this time interval, protamines play an important role in post-transcription and epigenetics. In addition, a subset of protamine mRNAs are never translated and remain in mature sperm cells, which is inherited in the zygote after fertilization [20]. Previous studies have shown that miRNA-122a, through binding to its complementary sequences in the 3' untranslated regions (UTRs) of the transition protein 2 (Tnp2) mRNA, decreases the target transcription[21]. P1, P2 and Tnps have the same promoter thus, their transcription occurs simultaneously[22,23]. On the other hand, miRNA-122 is likely to have similar effects on the protamine genes based on miRNA base data. Although the effect of the drug on infertility has been reported, its mechanism and molecular changes remain unclear. In this study, the correlation among miRNA-122 and protamine gene and protein expression levels were studied alongside semen analysis, and miRNA-122's association with protamine and seminal parameters were investigated.

PATIENTS AND METHODS

Study population

The medical ethics committee of Iran University of Medical Science approved this study (code: IR.IUMS.rec.1394.9211313202). In case control study, 24 men with normal semen analysis [according to World Health Organization (WHO) 2010 criteria] and 24 heroin-addicted men whose addiction were confirmed by a psychiatrist and experiments, were considered as the control and addiction groups, respectively. All volunteers executed the written informed consent which was conducted according to the Declaration of Helsinki and a questionnaire containing exact demographic information.

Inclusion and exclusion criteria

All participants were 20-50-year-old men with normal body mass index (BMI). Other criteria included normozoospermia, in the control group, and only heroin consumption for at least four months and collecting samples before initiating drug-tapering treatment protocols, in the addiction ones. Subjects with infertility, AIDS and hepatitis, and alcohol consumption were excluded from the study. Also, if addicted men consumed other narcotics in the last four months, they would be excluded from the study.

Sampling and preparation

Semen samples were collected by masturbation in sterile containers after 2 to 5 days of sexual abstinence and immediately sent off for processing according to WHO (2010) guidelines. A part of the samples was cryopreserved for nuclear protein evaluation. The rest of the samples were incubated to liquefy at 37 °C for 30 minutes. Gradient-swim up technique was used to precisely separate spermatozoa from seminal plasma. The pellet

Table 2. Demographic, semen analysis, and molecular data in the participants.

	Parameter	Control group Mean ± SD (n)	Addicted group Mean ± SD (n)	*p*-value
Demographic data	Age (year)	34.41 ± 1.25	34.87±1.80	0.836
	BMI (kg/m^2)	27.75 ± 0.88	22.30 ± 0.36	0.001
Seminal parameters				
Semen physical parameters	Volume (ml)	3.90 ± 0.35	3.22±0.35	0.136
	Semen pH	7.79 ± 0.06	7.58±0.06	0.003
	WBC	1.69 ± 0.41	8.61±1.73	0.001
Sperm concentration (×106/ml)		136.18 ± 25.23	146.22±37.32	0.823
Sperm motility (%)		65.51±2.57	41.96 ± 3.58	0.001
Sperm viability (%)		87.41±1.00	71.50 ± 4.59	0.002
Normal morphology (%)		16.98±3.97	12.48±1.49	0.300
Molecular data	Protamine-2 expression level (2-ΔΔCT)	3.59 ± 0.94	0.05 ± 0.02	0.002
Relative density of protamine-2 protein		0.27 ± 0.06	0.10 ± 0.02	0.017
miR-122 expression levels (2-ΔΔCT)		1.52 ± 0.54	3.51 ± 0.73	0.034

Table 3. Partial correlation between study variables.

		Addiction	Seminal pH	Seminal WBC	Sperm motility	Sperm viability	PRM-2	P2	miRNA-122
Addiction	Correlation	-	-0.362	0.391	-0.416	-0.315	-0.562	-0.382	0.440
	p value		0.020	0.011	0.007	0.045	0.002	0.144	0.019
Seminal pH	Correlation	-	-	-0.350	0.234	0.043	0.190	0.008	-0.302
	p value			0.025	0.141	0.790	0.343	0.975	0.118
Seminal WBC	Correlation			-	-0.467	-0.097	-0.267	-0.225	0.471
	p value				0.012	0.547	0.179	0.402	0.011
Sperm motility	Correlation				-	0.832	0.437	0.457	-0.404
	p value					0.001	0.023	0.075	0.033
Sperm viability	Correlation					-	0.288	0.378	-0.533
	p value						0.145	0.149	0.004
PRM-2	Correlation						-	0.772	-0.391
	p value							0.001	0.050
P2	Correlation							-	-0.309
	p value								0.244
miRNA-122	Correlation								-
	p value								

This correlation was adjusted for cigarette smoking in the addicted group.

and supernatant of each sample were also subsequently used for total RNA extraction and miRNA assessment.

RNA extraction and cDNA synthesis

The spermatozoa RNA was prepared using RNeasy Mini Kit (Qiagen, Germany). RNA 260/280 OD ratio and concentration were evaluated by nanodrop. cDNA was synthesized with RT primers according to the manufacturer's instructions (QuantiTect Reverse Transcription Kit, Qiagen, Germany).

Real-time semi-quantitative PCR technique

The PRM-2 (NM_001286356.1) expression level was determined by QuantiNova SYBR Green PCR Kit (Qiagen, Germany) and were normalized with Beta-actin gene. The primers for target genes are presented in **Table 1**. The temperature cycles (n = 45) were generally performed at 95 °C for 5 s and 59 °C for 30 s.

Total RNA isolation from seminal plasma

After thawing, seminal plasma aliquots were centrifuged twice (1,600 g for 10 min, then 16,000 g for 10 min) at 4 °C to harvest cell-free seminal plasma. The supernatant was carefully collected for subsequent assays.

For purification of cell-free total RNA, (primarily miRNAs and other small RNAs), from seminal plasma, we used miRNeasy Serum/Plasma Kit (Qiagen, Germany) according to the manufacturer's recommendations. The purity and integrity of RNA were checked by a 260/280 nm ratio measurement.

cDNA synthesis and real-time semi-quantitative PCR for miRNA

The miScript PCR System (Qiagen, Germany) was used for cDNA synthesis and determination of miRNA-122 expression levels. This system consists of the miScript II RT Kit for cDNA synthesis with oligo-dT primers, Ce_miR-39_1 miScript Primer Assay to monitor miRNA purification and amplification, and miScript SYBR Green PCR Kit to enable quantification of miRNA-122 by real-time sqPCR. Expression values were normalized with C. elegans miR-39 mimic as a reference gene.

Nuclear protein extraction

10 million spermatozoa from each sample were individually centrifuged at 3000 g for 5 minutes at room temperature (RT), and the pellet was washed three times with PBS (500 µl each time). The pellet was finally resuspended in 300 µl PBS and was sonicated for 5 minutes under these circumstances: 80 % strength, pulse on 15 seconds and pulse off 5 seconds. Then the samples were boiled for 30 minutes at 95 °C. After centrifugation at 15294 ×g for 20 minutes at 4 °C, and discarding of supernatant, 200 µl lysis buffer (0.2 M Tris-HCl, pH 7.5, containing 1 % SDS, and 10 % glycerol) was added and pipetted. Finally, after freezing and thawing of the specimens in liquid nitrogen five times, samples were centrifuged at 15294 ×g for 20 minutes at 4 ºC and the

Table 4. Simple linear regression analysis for sperm motility.

parameters	Unstandardized Coefficients		Standardized Coefficients	*p*-value
	B	Std. error	Beta	
Age (year)	0.137	0.395	0.052	0.731
BMI (kg/m2)	-0.772	0.756	-0.175	0.314
Cigarette smoking	-5.929	6.954	-0.158	0.400
Heroin addiction (yes or no)	-24.794	11.148	-0.672	0.033
Duration of opioid dependence (year)	-0.478	0.410	-0.224	0.252
Duration of heroin dependence (year)	0.259	0.662	0.070	0.699
Amounts of heroin consumed (mg/day)	4.168	4.394	0.185	0.349

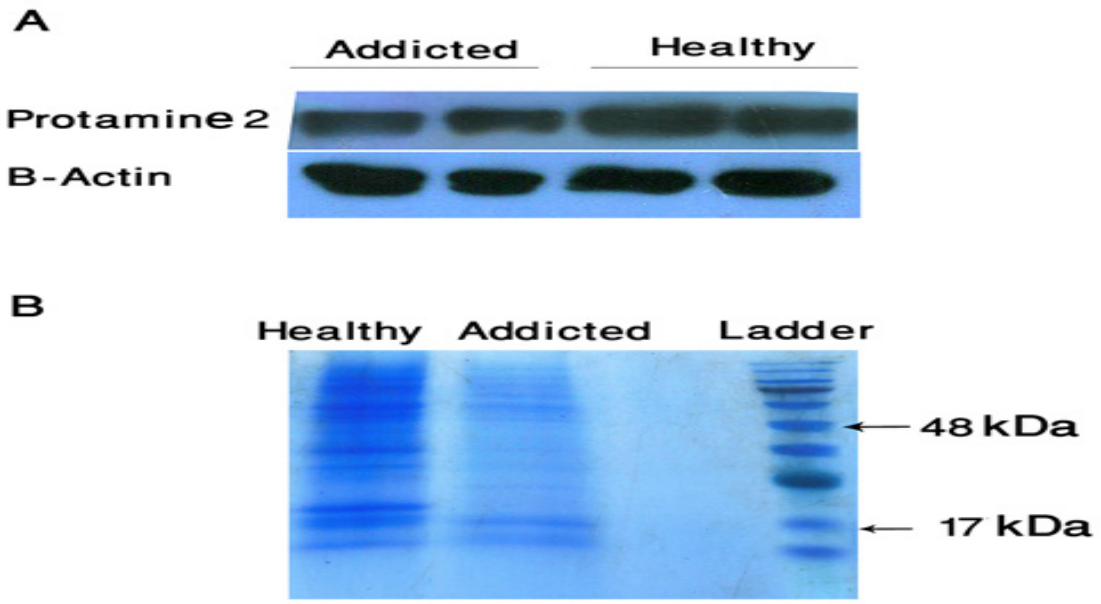

Figure 1. Analysis of protamine 2 (P2), beta actin (β-act). (A) Western blot, using an antibody specific for P2 and an antibody specific for (β-act). (B) Nuclear proteins extracted from spermatozoa, separated on a polyacrylamide gel and stained with Coomassie Blue.

supernatant was collected. Extracted protein was concentrated by a concentrator machine and protein content of each sample was calculated by the BCA method (Thermo Scientific™ Pierce™ BCA™ Protein Assay Kit, USA).

Western blotting

An extracted nuclear protein from each sample was separated by 15 % polyacrylamide SDS-PAGE. After electrotransfer onto a PVDF membrane, blocking was performed in buffer containing 5 % non-fat dry milk in 4 ºC overnight and the membranes were washed with 0.05 % Tween 20 in PBS.

Proteins were detected using anti-protamine 2 antibody (ERP15738, Abcam; MV= 17 kDa) and anti-beta-actin antibody (ab8227, Abcam, MW=41.7 kDa) as primary antibodies, and goat anti-rabbit IgG H&L (HRP) (ab6721, Abcam) as the secondary antibody and were visualized with the ECL reagent (Amersham, Canada) according to the manufacturer's instructions. The primary antibodies were diluted 1:1,000 in 2 % non-fat dry milk (Biolife) in PBS. The level of protamine was quantified via densitometry and normalized to beta-actin protein levels.

Statistical analysis

Statistical analysis was performed using a statistical

software package

SPSS (Ver. 16.0, Chicago, SPSS Inc.). The parametric distribution was evaluated with Kolmogorov- Smirnov test. The differences between groups were statistically determined by Chi-square, independent t-test, and Mann-Whitney test. The binary and multiple regression analyses were performed among gene and protein expression levels and other parameters. 2-ΔΔCT values were used to compare the target gene expression levels. *p*-value < 0.05 was proposed to be significant. The alphaEasseFC software was also used to determine the protamine-2 density versus β-actin.

RESULTS

Demographic and semen analysis information of all participants is shown in Table 2. There was no significant difference in the mean age between the 2 groups (34.41 ± 1.25 vs. 34.87 ± 1.80). Although BMI was in the normal range in both groups, this parameter in the addicted men (22.30 ± 0.36 kg/m^2) was significantly lower than the healthy men (27.75 ± 0.88 kg/m^2) ($p \leq 0.01$). All subjects in the addicted group smoked cigarettes, so this factor was statistically different between the two groups ($p \leq 0.01$). There was no significant difference in semen volume (3.90 ± 0.35 vs. 3.22 ± 0.35), sperm concentration (136.18 ± 25.23 vs. 146.22 ± 37.32) or normal morphology (16.98 ± 3.97 vs. 12.48 ± 1.49) between healthy and addicted men. However, seminal pH (7.79 ± 0.06 vs. 7.58 ± 0.06), white blood cells (WBCs) in semen (1.69 ± 0.41 vs. 8.61 ± 1.73), and sperm viability (87.41 ± 1.00 vs. 71.50 ± 4.59) and motility (65.51 ± 2.57 vs. 41.96 ± 3.58) were significantly altered in the heroin consumption group.

Protamine-2 gene and protein content and miRNA-122 expression

PRM-2 concentrations that was given by real-time sqPCR were significantly decreased in addicted men versus healthy ones (3.59±0.94 and 0.05±0.02, respectively; $p \leq 0.01$). In western blots of sperm nuclear proteins separated by SDS–PAGE (Fig 1 A, B), increased density in P2 content was also observed in the addicted group (0.27 ± 0.06) as compared to the healthy group (0.10 ± 0.02) ($p \leq 0.05$) (**Table 2**). In addition, the real-time sqPCR analysis revealed that miRNA-122 expression levels were increased in addicted men as compared to the healthy group (3.51 ± 0.73 versus 1.52 ± 0.54) ($p \leq 0.05$).

Correlation analysis

Cigarettes were smoked by all participants in the addicted group, but not by participants in the healthy group, so partial correlation was used to eliminate that variable. The correlation results have been presented in Table 3. WBC count in seminal plasma was negatively correlated with seminal acidity and sperm motility. However, there was a positive correlation between the presence of WBC and miRNA-122 expression levels in seminal fluid. The increase in miRNA-122 is associated with a decrease in motility and survival rate of spermatozoa and a reduction in the amount of PRM-2.
Among the groups, a positive significant relationship between sperm cell motility and spermatozoa survival rate, and PRM-2 expression levels was demonstrated. The number of copies of PRM-2 was directly correlated with the P2 content.

Simultaneous analysis of demographic variables on sperm motility by simple linear regression

Among the age, BMI, cigarette smoking, heroin addiction, duration of opioid and heroin dependence, and amounts of heroin consumed, only heroin addiction parameter affects sperm motility ($p < 0.05$, **Table 4**).

DISCUSSION

To the best of our knowledge, no study has been conducted to addiction science that simultaneously evaluates the conventional and molecular parameters associated with infertility. Main obstacles on the way of human studies can be legislation and ethical considerations as well as simultaneous polydrug abuse. Our findings can contribute to increase our knowledge about the seminal molecular changes in the addicted men.
Although BMI of heroin-addicted men was in the normal range, the mean of this variable in that group was statistically lower than the healthy one. Based on demographic data, the economic and educational condition of heroin consumers were different from healthy men. Based on the previous many human and animal studies, we conclude that a decrease in BMI in addiction cases [3,5,24,25]. Yilmaz et al. (1999) determined morphine reduced body weight with decreased metabolism caused by inhibition of androgen production along with the reduction of gastrointestinal activity[25]. Illicit drugs can compete with food for brain reward sites, and decrease appetite[26,27]). Based on these results, it would be expected BMI is a heroin-dependent variable and is considered as a physiological change in the drug users. It seems that one of the possible pathways for heroin effects on gametogenesis is with BMI reduction.
PH and WBC count are some of the seminal quality criteria, which decreased and increased in the addiction group, respectively. The results of partial correlations and previous studies suggests that the presence of WBC changes seminal pH leads to acidification of seminal fluid[28] and reduced survival rate and motility of sperm cells[29]. Similar to the link between heroin addiction and BMI reduction, leukocytospermia, which refers to the presence of leukocytes in semen, is one of the inherent heroin-related clinical manifestations. Leukocytes produce reactive oxygen species (ROS) in semen and contribute to male infertility [7,8,30]. Few studies have investigated sperm microenvironment in addicted cases. However, our finding is in line with Agrawal et al. (2014) and Nazmara et al. (2019) who reported leukocytospermia in addicted cases [5,8].
In this study, protamine-2 content (gene and protein) in ejaculated spermatozoa and miRNA-122 levels in seminal fluid showed that addiction could lead to protamine deficiency and alter the functionality of cell-free seminal RNAs. It seems that insufficiency of protamine-2 in the addicted group is dependent on epigenetic regulators. Data on the copy number of protamine-2 mRNA confirms this conclusion in three ways. First: the significant positive correlation between protamine-2 protein and mRNA levels could support the idea that diminished P2 content was due to the low levels of PRM2. Second: the negative correlation between PRM2 and miRNA-122 levels suggests that abnormally high levels of miRNA-122 may lead to protamine-2 transcripts would be inaccessible or non-functional ones. Third: an abnormally high level of miRNA-122 and its association with seminal acidification and leukocytospermia, along with the correlation of these factors with addiction, suggests that the increase of miRNA-122 may be affected by heroin-dependent seminal changes.
Protamine-2 is the most important protein involved in spermatozoa chromosome condensation[31]. It is said that a defect in protamine gene expression is not due to gene mutations and may reflect new transcription regulations or incomplete post-translational processes [32]. As previously mentioned, protamine transcription and translation is temporally uncoupled during spermiogenesis[33]. Although this time-separation is critical to the sperm development and is transcriptionally gene silent, it can make protamine mRNAs sensitive to intra- and extra-cellular alterations which result in protamine deficiency. The most important hypothesis for reducing protein in a condition where the level of gene expression is low, (as in the present study), or normal is summarized: (a)Abnormalities in the post-translational process of P2: Defective protein kinases and their activating pathways were reported in patients with diminished P2 concentration by Aoki and Carrell (2003)[34] and Wu et al. (2000)[35]. (b) Reduction in PRM2 levels: in mice, miRNA-469 binds to the coding regions of Tnp2 and PRM2, and hence represses those protein expressions at the translation level with minor effects on mRNA degradation[23]. Furthermore, inhibition of Tnp2 transcription, (one of the most widely studied genes in humans), was achieved by binding miRNA-122 to 3'-UTR of Tnp2 and its endonucleolytic cleavage activity in sperm-like cells[22]. These are two examples of post-transcriptional regulation by miRNAs and our findings were consistent with the reports by the aforementioned investigators.
The presence of protamine-2 transcripts in mature spermatozoa RNA profiles and its transfer to the oocyte during fertilization[36] indicates the importance of the protamine-2 mRNA as a gene expression regulator in those cells. In other words, PRM2 not only contributes to toroidal structure, but also can be considered as one of the biomarkers which guarantees successful fertilization.
Motility was another modified parameter in the addiction group which was measured and reported by computer-assisted sperm analysis (CASA). Considering that PRM2 and miRNA-122 levels had significant correlations with sperm motility, it is legitimate to speculate that heroin abuse affects chromatin packaging, expression of motility-related genes, and sperm viability, resulting in asthenospermia. Up-regulation of

miRNA-122 in patients with asthenozoospermia[14] decreased motility with increased % DFI (DNA Fragmentation Index) in opiate users, especially heroin-addicted men[37], depression of sperm motility along with movement-related gene impairment, including Catsperes, in mice that were addicted to Iranian kerack[24], are part of the studies which have similarities with our study.
Semen and sperm cells are a major source of endorphins and enkephalins[38], and their defined activity levels are required to regulate sperm motility[39]. However, heroin as a μ-agonist decreased sperm motility[40]. In view of cigarette smoking is the most confounding factor affecting sperm mobility[41], and all participants in addicted group smoking cigarettes, simple linear regression was used to explore the most important demographic factors affecting mobility. Our findings showed that among the studied demographic variables, heroin addiction has the most deleterious effect on sperm motility.
Our recommendations for further research are: Evaluation of other factors associated with nuclear condensation; In vitro assessment of the impact of heroin on ejaculated semen; The study of sperm surface receptors which are necessary in sperm-oocyte attachment.

ACKNOWLEDGEMENTS

The research team would like to thank the individuals who participated in this study. This study was supported by Iran University of Medical Sciences (IUMS), (Number: 94-02-30-25973) and INSF (number: 94017358).

FUNDING

This study was funded by a grant from Iran University of Medical Sciences (IUMS), (Number: 94-02-30-25973) and INSF (number: 94017358) for a Ph.D. student thesis.

CONFLICT OF INTEREST

No potential conflict of interest was reported by the authors.

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