Original Article

Assessment of Adiponectin and Sperm Function Parameters in Obese and Non-Obese: A Comprehensive Study

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Abstract

Objective: The role of adiponectin in sperm function is inconclusive and there is a paucity of evidence. Obesity shows an ambiguous influence on sperm motility, and male subfertility. The aim of this study was to compare the role of adiponectin and sperm functional parameters among obese and non-obese men.

Materials and Methods: In this comprehensive study, 64 male patients were included, and were classified as non-obese [body mass index (BMI)< 24.9 kg/m2, n=32] and obese (BMI >25 kg/m^2, n=32) groups. Sperm analysis, was conducted using World Health Organization (WHO) 2010 standards. Real-time polymerase chain reaction (PCR) and enzyme-linked immunosorbent assay (ELISA) were used for the analysis of adiponectin gene expression and protein levels, respectively. Sperm viability was assessed using the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT test), Acridine orange (AO) test was utilized to detect DNA denaturation, and sperm chromatin dispersion (SCD) technique was used to investigate the fragmentation of DNA.

Results: In obese men, adiponectin gene expression (P<0.0001) and protein levels (P<0.001) were significantly lower compared to the non-obese group. Additionally, sperm motility, was significantly lower in the obese group. The rapid progressive (RP) motility was less in obese men in comparison to the non-obese group (P<0.001). Sperm count and morphology were not significantly different in the two groups. DNA denaturation and DNA fragmentation were significantly more frequent in the obese group than in non-obese men (P<0.05) and (P<0.01), respectively. The obese men showed significantly lower sperm viability compared to the non-obese group (P<0.05).

Conclusion: This study showed no significant correlation between the evaluated variables (sperm parameter, sperm viability, DNA fragmentation and integrity), and obesity in men. Based on these results, adiponectin may potentially play positive role in sperm function for acquiring fertility.

Keywords: Adiponectin, Chromatin, Obesity, Sperm, Viability

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Introduction

Adipose tissue has a complex system, consisting of adipocytes, pre-adipocytes, fibroblasts, endothelial cells, immune cells such as macrophages, dendritic cells, and T cells, which secrete fat metabolites, cytokines, and adipokines. It maintains energy homeostasis by preserving proteins, controlling energy balance, fertility, and inducing an increase in immunological response. However, excess fat causes imbalance with a detrimental effect on health. Adipose tissue is a toxic depot of triglycerides, and is a vital endocrine organ releasing adipokines, whose actions of mechanism have paucity of evidence (1). The role of adipokines is to preserve energy homeostasis, having other endocrine axes with direct influences on various organs, which are emerging with the advent of research in these realms (2). Metabolic disorders, like obesity, can jeopardized adipose tissue hormonal milieu, effecting health factors (3).

Obesity is a serious global health problem including multifactorial diseases, like atherosclerosis, hypertension, diabetes, cardiovascular disease (CVD), stroke, infertility, and cancer, potentially leading to one´s death (4-6). The body mass index (BMI) is indicative of the state of body weight (7). BMIs of 25-30 kg/m^2 and values higher than 30 kg/m^2 are considered as obese. Over the past decades, obesity has become a public health priority in both genders in Iran (8), with a risk of male-factor infertility, suggesting prevention and management due to the comorbidities, oxidative stress (OS), and complications (9). The evidence of decreased male fertility ,has encouraged researchers to unveil the link between metabolic disorders like obesity and male fertility (10). The issue of male infertility has become a global public health problem, indicating the important effects of obesity on sperm functions (11). In a previous study, non-obese men showed higher sperm function, and lower DNA fragmentation index (DFI) (12) than obese men. Obesity has shown to decrease adiponectin levels, serum and intra-testicular levels of follicle stimulating hormone (FSH), luteinising hormone and testosterone, sperm count, motility, viability and normal morphology, but increase serum leptin, epididymal malondialdehyde level and sperm DNA fragmentation (13).

Adipose tissue, produces many bioactive polypeptides called adipokines, inducing metabolic disorders and infertility (14), suggesting the missing link between obesity and infertility (15). Adiponectin is a specific adipokines, found in adipose tissue (16). Unlike other adipokines, serum adiponectin levels shows an inverse relationship with visceral fat and body mass (17). Adiponectin and its receptors (AdipoR1 and AdipoR2) have been reported in various male genital cells of different species, like chickens, mice, rams, cattle and humans (18). It has been reported that diet-induced obesity in mice, leads to changes in serum levels of the hormone adiponectin, but does not lead to epigenetic changes in a high-fat diet mouse (19).

The exact role of adiponectin in diagnosis of male fertility is inconclusive at this point, but there is a clear association. Previous studies have investigated the function of adiponectin receptors on testicular cells. However, to our knowledge, no study has reported the role of adiponectin on sperm molecular factors and DNA integrity in men. Similarly, the correlation between seminal adiponectin concentration and sperm function parameters in patients in Isfahan, Iran, has not been reported. Thus, there is a need to explore the adverse effects of obesity and the possible role of adiponectin in sperm function in our population. Consequently, this study was considered as a basis of novelty to explore the impact of adiponectin on normal reproductive sperm function and its possible role on infertility. To test these hypotheses, we studied the *adiponectin* gene expression and protein level in human semen, and its relationship with sperm function in obese and non-obese normozoospermic men.

Materials and Methods

Study population

This comprehensive study, was approved by the Institutional Ethics Committee of Isfahan University of Medical Sciences (IR.MUI.MED.REC.1398.568). Informed permission was acquired from all normozoospermic men referred to the Andrology Unit of Hazrat-e-Maryam Fertility and Infertility Center of Shahid Beheshti Hospital, Isfahan University of Medical School, Isfahan, Iran. According to World Health Organization (WHO) criteria published in 2010 (20), sperm samples were classed as normozoospermic. Based on the BMI values, sperm and blood samples of 64 men were collected and separated into two groups: obese and non-obese normozoospermic males.

Inclusion criteria

The age of the participants was between 25 to 55 years. The BMI values ranging from 24.8 to 18.9 kg/m^2 were considered as non-obese men, and those higher than 25 kg/m^2 were classified as obese men. The participants were told to refrain from sexual activity for at least 3 to 4 days before the experiment.

Exclusion criteria

Individuals with a history of cryptorchidism, varicocele, vasectomy, drug abuse and usage of exogenous hormones (replacement therapy of testosterone) were excluded from the study. Biochemical variables, like normal serum inhibin B<50 ng/ml, HBA1c ≥ 6.5%, normal weight with triglycerides> 2.3 mmol/ml, and FSH level>12.4 IU/ml were also excluded.

Fertility assessment

Fertility was evaluated through sperm analysis, blood sampling, physical examination and a general questionnaire. The questionnaire included the body weight, height, medical history, lifestyle variables, use of cigarettes and other narcotics, alcohol, drugs and any nutritional supplements. Fresh semen samples were collected by masturbation in sterile containers and were evaluated using computer-assisted sperm analysis (CASA) in accordance with WHO (2010) criteria (21).

Sperm preparation and analysis

After liquefaction, semen samples obtained in sterile containers were analyzed for the volume as well as, sperm morphology, and motility, according to the WHO (2010) guidelines. The sperm count, morphology, and motility were evaluated using a CASA-system (CASA, VT-Sperm Test.2.3 model-company of Video Test-Finland) guidelines. Each semen sample was washed twice and re-suspended in modified Hams F10 with 5% human serum albumin (Irvine Scientific, Santa Ana. California) (22). Sperm viability was determined using the MTT Test. DNA fragmentation and denaturation were assessed by means of the sperm chromatin dispersion (SCD) technique and the AO test. The adiponectin level was assessed by ELISA and RNA isolation for gene expression analysis was performed using real-time quantitative reverse transcription-polymerase chain reaction (RT-PCR).

Sperm motility assessment

The percentage of motile spermatozoa was studied in obese and non-obese samples by evaluation of the samples under a light microscope (Olympus, Tokyo, Japan) equipped with CASA system (CASA, VT-Sperm Test, 2.3 model- Company of Video Test-Finland). Percentage of rapid progressive (RP), progressive (P), non-progressive (NP) and immotile spermatozoa (IM) were assessed in both groups. FP sperm cells are those that swim forward in a straight line. SP sperm swim forward, but either in linear or curved line. NP sperm move their tails but do not move forward and IM sperm do not move at all. The results of sperm motility were evaluated compared between two groups of sperm samples (23).

Gene expression by real-time polymerase chain reaction

Total human sperm RNA was extracted as per the

manufacturer's protocol of Super RNA extraction kit (Thermo-Fisher, USA). Sperm samples were stored at -80˚C until RNA extraction. Briefly, 800 µl of lysis buffer was added to the sperm sample and vortexed. Next, 300 µl of chloroform (Merck, Germany) was added to the sample and vortexed for an additional 15 seconds. After that, it was incubated for 5 minutes at room temperature. The sample was centrifuged at 12,000 rpm at 4˚C for 5 minutes, forming three phases in the microtube. The supernatant fluid was removed, and 300 µl of sperm cell precipitate was added and vortexed. The sperm cells were transferred to the column, and again centrifuged at 12,000 rpm for 30 secs at room temperature. 700 µl of PBS was added and centrifuged at 12,000 rpm for 90 seconds at room temperature. The column was then placed in a new sterile microtube and 35-45 µl of elution buffer was added, then placed in thermomixer at 65˚C temperature for 3 minutes, and centrifuged at 12,000 rpm for 2 minutes at room temperature. The final concentration and RNA purity were measured at 260 nm using a spectrophotometer (Nano-drop, Eppendorf, Germany). Subsequently, total RNA was reverse transcribed to complement deoxyribonucleic acid (cDNA) using cDNA Synthesis Kit (Anacell, Iran), according to manufacturer's instructions. The primers, which are summarized in Table 1, were designed on the National Center for Biotechnology Information (NCBI) site and certified by Blast Primer software. Each primer's final concentration was 1 µM. Real time polymerase chain reaction (PCR) was run on Life Cycler 96 system (Roche Diagnostics, Gmblt, Germany) by the use of SYBR green PCR kit (Anacell, Iran). The PCR conditions included 95˚C for 15 minutes followed by 40 cycles at 95˚C for 20 seconds, 60˚C for 30 seconds and 72˚C for 30 seconds. Finally, for data acquisition, the samples were placed at 72ºC-95ºC temperature for 0.5ºC/0.05 seconds. Glyceraldehyde-3-phosphate dehydrogenase *(GAPDH)* gene was used as the housekeeping gene. The relative expression of messenger RNA (mRNA) was specified via the $2^{-\Delta\Delta CT}$ method.

The concentration of adiponectin by ELISA method

The adiponectin concentration in 32 obese and 32 non-obese samples was evaluated by human adiponectin enzyme-linked immunosorbent assay (ELISA) kit (Mediagnost human Adiponectin ELISA kit, E09), which was performed per the protocol of the manufacturer. Quality control and semen were diluted 200 times with dilution buffer before testing, ideally in two phases. The coefficients of inter-and intra-assay variation were determined to be less than 6.7 and 4.7%, respectively. Within 30 minutes, absorbances were measured at 450 nm using an ELISA Microplate Reader RT-6000, lorderan with >590 nm as the reference wavelength.

Sperm viability by MTT method

Using the MTT test, we determined sperm viability in 32 obese and 32 non-obese samples. This experiment was done through a technique outlined by Mosmann (24). In summary, 10 µl of MTT (Sigma, USA) stock solution (5 mg/ml ham's F10) was poured into each tube of sperm suspension. The tubes were then put in an incubator for 1 hour at 37˚C. After the tubes were centrifuged for 6 minutes at 6000 rpm, the precipitate was dissolved in a solution of 200 µl dimethyl sulfoxide (DMSO), which was centrifuged for 4 minutes at 4000 rpm. 100 µl of the purple solution was poured into a 96-well plate, and its absorbance rate was measured at 505 nm through an ELISA reader (RT-6000 | lorderan). The sample's optical density was then utilized to specify the percentage of viable sperm (25).

Acridine orange test

The Acridine orange (AO) stain is used to measure the rate of DNA denaturation. The smears of 32 obese and 32 non-obese samples were produced and air-fixed for 20 minutes before being fixed in a Carnoy's solution (methanol/glacial acetic acid at a ratio of 3:1) at 4˚C for at least 2 hours. The prepared samples were then stained with a newly produced AO solution (at a 0.19 mg/ml concentration in Mc Ilvain phosphate citrate buffer (pH=4). On the same day, each smear was examined through a fluorescencet microscope, with a 460 nm filter. 200 spermatozoa were analyzed in each slide, and the percentage of healthy sperm cells that had double-stranded DNA (normal green fluorescent) was determined while single-stranded DNA was detected through red fluorescence (abnormal cells) (26, 27).

Table 1: Sequence of primers used to synthetize the studied genes

Oligo gene	Primer sequence (5´-3´)	Tm (˚C)	MW (g/mol)	GC content (%)
ADIPONECTIN	F: ACTGCAGTCTGTGGTTCTGA	57.3	6139	50
	R: GAGTCGTGGTTTCCTGGTCA	59.4	6155	55
GAPDH	F: AAGCTCATTTCCTGGTATG	52.4	5794	42.1
	R: CTTCCTCTTGTGCTCTTG	53.7	5398	50

Tm; Temperature and MW; Molecular weight.

Sperm chromatin dispersion method for DNA fragmentation detection

SCD test was employed to detect DNA fragmentation by using a halo sperm kit (Idevarzan-e-Farda Co., Tehran, Iran). The 32 obese and 32 non-obese sperm samples were evaluated according to manufacturer's protocol. Each sample was washed twice in phosphate-buffered saline (PBS), before adding 50 µl of the samples to the agarose. Then placed 30 microliters of the samples on a glass slide, wrapped in foil, and refrigerated for 5 minutes. Denaturation and slip solutions were applied to the slides. Before dehydrating with a rising gradient of ethanol (70%, 90%, and 100%), each sample was rinsed with distilled water for 5 minutes. The samples were washed and dried. On each plate, 200 sperm cells were inspected under a light microscope for halo analysis (1000x magnification). Sperms without, or with small, halos were taken as having fragmented DNA while sperms with medium/large halos were thought to have intact DNA (28).

Statistical analysis

SPSS (version 20, IBM Corporation, Armonk, NY) was used for conducting statistical analysis. Kolmogorov–Smirnov test was used to check the normality of the data. The student t test was utilized to compare the acquired results between the non-obese and obese groups. Pearson's correlation test was used to study the relationship between variables. Data were analyzed as the means and standard deviation (mean ± SD), and $P<0.05$ was considered as statistically significant.

Results

The comparison of sperm parameters by CASA

Sperm rapid progression (fast motility) in obese group (11.70 ± 6.99) was significantly different from non-obese group (18.25 ± 6.69, $P<0.001$, Fig.1A, B) and sperm progression (slow motility) in the control group (35.94 ± 8.55), was not significantly different from the obese group (32.0 ± 7.57). Additionally, sperm morphology in the non-obese group (4.83 ± 0.89) compared to the obese group (4.78 ± 0.73) was not significantly different ($P>0.05$). Similarly, the sperm count in the non-obese group (122.1 ± 34.13) compared to the obese group (115.0 ± 33.21) was not significantly different ($P>0.05$, Fig.1C, D).

Comparison of adiponectin levels in obese and non-obese individuals

The seminal fluid adiponectin levels in the obese group (5.42 ± 0.21 ng/ml) was significantly different from non-obese group (6.13 ± 0.76 ng/ml, $P<0.001$, Fig.2A).

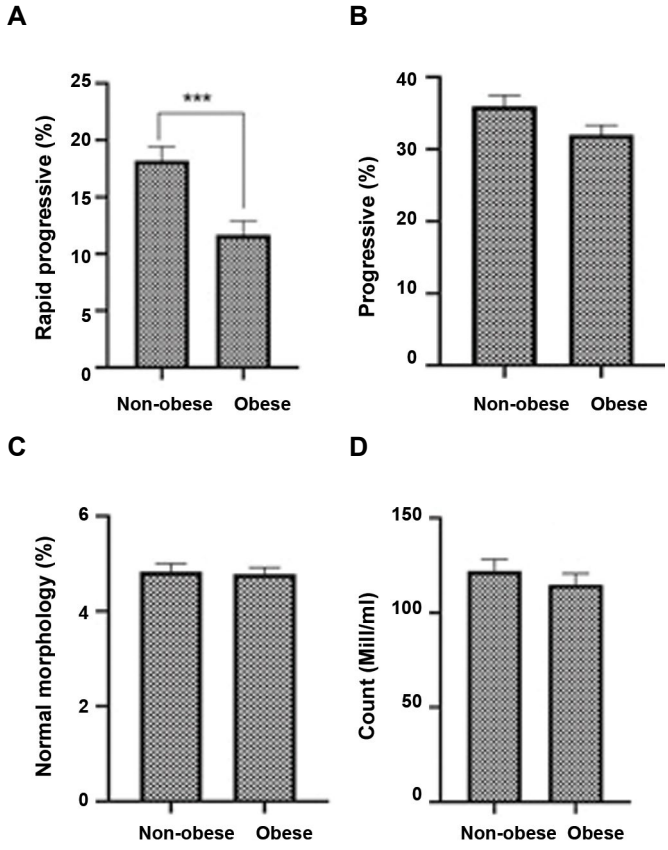

Fig.1: Percentage of sperm parameters (mean ± standard deviation) in non-obese and obese groups. Sperm motility, **A.** Rapid progressive and **B.** Progressive. **C.** Sperm morphology and **D.** Count percentages. ***; $P<0.001$.

Comparison of *ADIPONECTIN* gene expression in obese and non-obese individuals

The expression level of *ADIPO* gene was evaluated in seminal specimens taken from non-obese and obese patients. The obese group (0.44 ± 0.060) was significantly different from the non-obese group (1 ± 0, $P<0.0001$, Fig.2B).

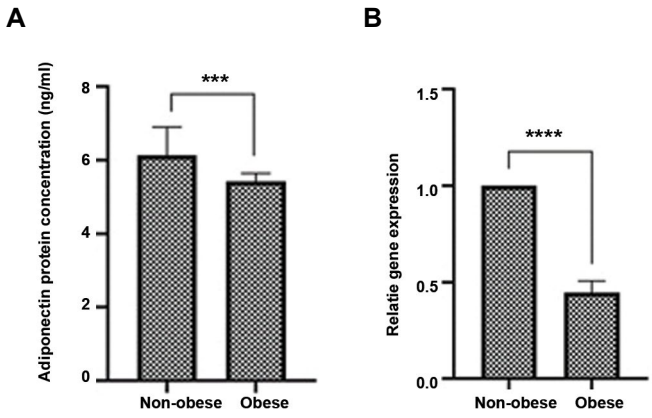

Fig.2: Evaluation of semen adiponectin protein concentration and relative gene expression (mean ± standard deviation) in obese and non-obese groups. **A.** Adiponectin protein and **B.** Gene expression. ***; $P<0.001$ and ****; $P<0.0001$.

MTT assay

Sperm viability was analyzed by MTT assay. The proportion of viability in the obese group (0.48 ± 0.27) was significantly lower from the non-obese group (0.65 ± 0.17, P<0.05. Fig.3A).

Acridine orange test

According to our findings, the rate of sperm DNA denaturation in the non-obese group (52.00 ± 15.85) was significantly lower from the obese group (67.35 ± 24.05, P<0.05, Fig.3B).

Evaluation of DNA fragmentation

Using the SCD technique, we discovered that the proportion of sperm with fragmented DNA in the non-obese group (17.91 ± 9.96) was significantly different from the obese group (25.83 ± 9.56, P≤0.01, Fig.3C). The SCD method revealed that obesity has a detrimental effect on the integrity level of sperm DNA, while the non-obese group compensates for obesity's negative effect on the DNA of sperm (Fig.4).

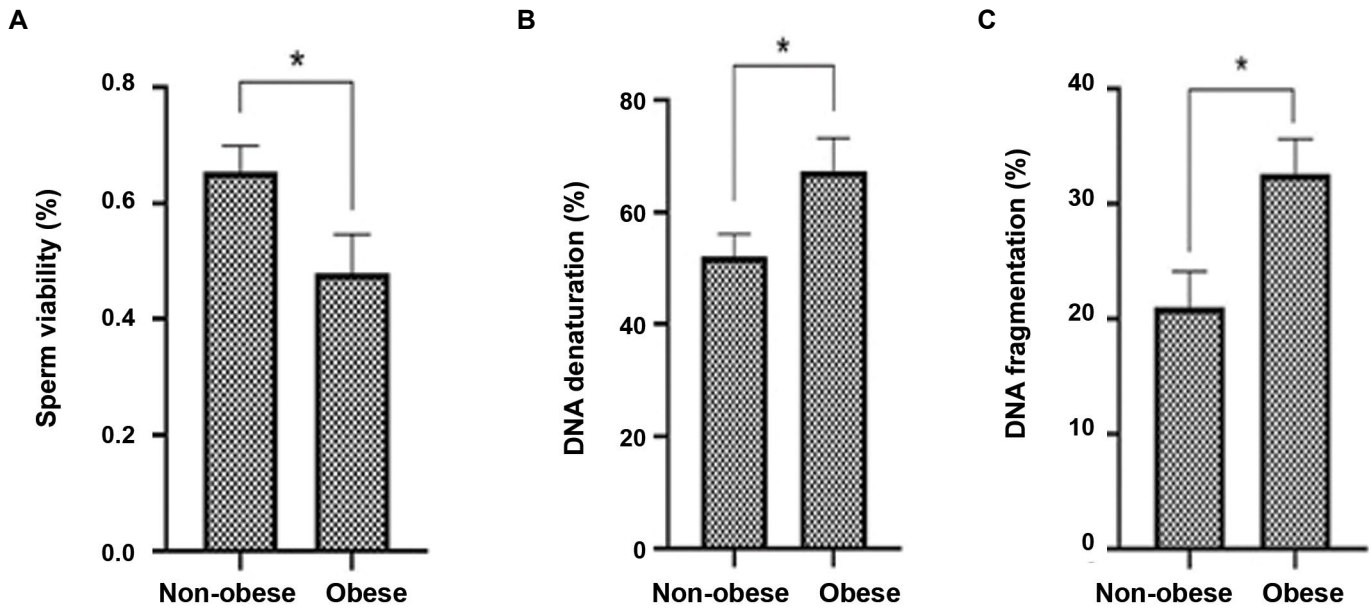

Fig.3: Percentage of sperm viability, DNA denaturation and DNA fragmentation (mean ± standard deviation) in obese and non-obese groups. **A.** Sperm viability, **B.** DNA denaturation (*; P<0.05), and **C.** DNA fragmentation (*; P≤0.01).

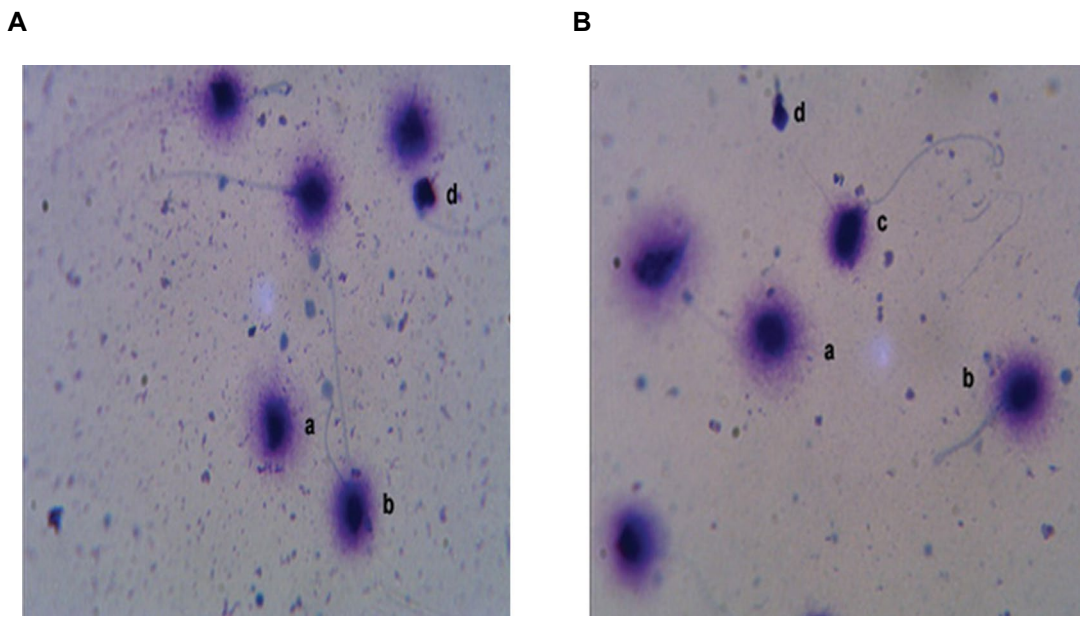

Fig.4: Percentage SCD test. Large halo sperm (a), medium halo sperm (b), small halo sperm (c), and no halo sperm (d). Sperm cells with large/medium halos have healthy DNA, sperm with small or absent halos had damaged DNA (light microscopy, magnification 1000x). **A.** Non-obese men and **B.** Obese men (n=32).

Correlation of variables

Pearson's correlation analysis revealed no significant relationship between adiponectin and sperm parameters, viability, fragmentation, and denaturation of DNA in the obese or non-obese group (Table 2).

Table 2: Pearson's correlation coefficient between research variables

Variable	Obese (n=32)	Non-obese (n=32)
Sperm count	r=-0.256	r=-0.212
	P=0.196	P=0.356
Morphology	r=0.277	r=-0.033
	P=0.297	P=0.886
Progressive	r=-0.061	r=0.248
	P=0.819	P=0.278
Rapid progressive	r=0.116	r=0.161
	P=0.562	P=0.483
Viability	r=-0.130	r=0.022
	P=0.628	P=0.935
Acridine orange	r=-0.052	r=0.016
	P=0.845	P=0.953
SCD test	r= 0.409	r=-0.126
	P=0.058	P=0.584

Discussion

Obesity was shown to have a deleterious influence on critical sperm parameters and DNA integrity in normozoospermic individuals in this research. Nevertheless, we did not discover any statistically significant correlation between adiponectin and the study factors. Obesity has more than tripled among men of childbearing age in the last 30 years, which may be a reason for the increased infertility and decreased sperm parameters.

On the other hand, the relationship between an increased BMI and sperm functions have been hypothesized extensively, yet there is a paucity of experimental evidence about these observations. In a study on 1558 males, 23.9% of men showed decreased sperm count when BMI was more than 25 kg/m^2 (29). Another study, reported a decreased motility of sperm in obese men compared to the group with normal BMI. The average value for sperm motility was 51.4% in non-obese men, while it was 46.6%, in men with BMIs higher than 30 kg/m^2 (30). A previous study reported that obesity was associated with lower number of motile sperms, and higher incidence of abnormal morphology. Our findings are in agreement with that study, suggesting a negative correlation between obesity and sperm quality and viability. However, in contrast to our results, another study did not find that obesity has detrimental influence on sperm morphology (31). It is reported that obesity could influence sperm quality, but the putative mechanism of action of this pathogenesis remains unclear. A causal impact of the adipokines profile that changes during obesity, can be the link between obesity and infertility (15). One study has found that the concentration of seminal adiponectin in ram sperm is related to sperm motility (32). In humans, it seems that seminal adiponectin concentrations is directly related to both sperm count and morphology (33), as overweight individuals show lower levels of adiponectin. As a result of the decreased level of adiponectin in these patients, the sperm count, motility, morphology and viability are found to be affected in obese men (34). Similarly, in our study, non-obese men had higher levels of *ADIPONECTIN* gene expression and protein than the obese individuals, showing the correlations of adiponectin with BMI, which is in agreement with previous findings (35).

Sperm cell viability showed a direct relationship with sperm fertility (36). In our study, the proportion of sperm viability in the non-obese men was significantly higher than the obese men, which is in line with a previous research study (37). Our study found that obesity, sperm parameters and viability can affect sperm function, as the non-obese men had significantly lower mean rates of sperm DNA denaturation and fragmentation compared to the obese group. Similar to our study, increased adiponectin levels in Holstein bulls, was reported to have an increased DNA fragmentation in another study (18). Our results, regarding the role of adiponectin, are in agreement with the above observation of adiponectin levels. Hypersensitivity of sperm to reactive oxygen species (ROS) and OS, induces DNA damage (38, 39). Sperm DNA denaturation and fragmentation was reported in obese people with high ROS levels. Sperm DNA damage, was reported to be associated with morphology and motility (39, 40). In our research, sperm motility in the non-obese group (RP and progressive) was greater than the obese group, which may be due to higher level of *ADIPONECTIN* gene expression and protein in the non-obese group confirming the beneficial effect of adiponectin. We observed that especially RP sperms in the non-obese men were significantly higher than those in the obese group. However, no significant relationship was seen between the count and sperm morphology, as the determined values were located within the standard range of WHO criteria. It was reported that damage to sperm DNA is not necessarily associated with change in sperm parameters. Finally, we found no significant correlation between adiponectin levels and viability of sperm cells, denaturation and fragmentation of DNA, or molecular characteristics of the sperm cells in the obese and non-obese groups.

Conclusion

Our results showed that obesity can play an important

role in male infertility as well as in has influence on sperm motility, viability, and DNA integrity in normozoospermic men. Although, we did not discover any statistically significant correlation between adiponectin and the research variables. However, further research is required to unveil more accurate information on the extent to which adiponectin may mediates sperm functions, to reduce the risk of male infertility. Such potential treatment may especially be useful in cases where infertility is related to obesity and metabolic syndromes. In light of the current knowledge, adiponectin could be suggested as a marker for sperm function, which can be determined in further research.

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Author' Contributions

G.R.D.; Contributed to concept and design of the study, and final approval of the manuscript, and responsible for overall supervision. G.R.D., M.P., M.Sh.; Performed all the experiments, analyzed the data and interpreted them. G.R.D., M.P.; Drafting the manuscript. All authors read, and approved the final version of the manuscript.

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