




# 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 disintegrated 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  $\mu$ l 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 ( $\times$ 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  $\mu$ l 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<sup>®</sup> was carried out following the guidelines of its developer (24). After determining the sperm concentration,  $2 \times 10^6$  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  $\mu$ l 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  $\mu$ M 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,  $5-10 \times 10^6$  sperm cells were mixed with 400  $\mu$ l 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  $\pm$  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 ( $42.8 \pm 15.56$  vs.  $59.7 \pm 13.38$ ,  $P=0.018$ ), and the HOS test ( $60.09 \pm 14.58$  vs.  $75 \pm 14.58$ ,  $P=0.025$ ) 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 ( $70.30 \pm 17.36$  and  $42.36 \pm 14.44$ ,  $P=0.001$ ), protamine deficiency ( $63.95 \pm 12.78$  and  $40.80 \pm 15.27$ ,  $P=0.002$ ), and sperm DNA damage ( $35 \pm 18.25$  and  $22 \pm 4.41$ ,  $P=0.044$ ) 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 ( $1.74 \pm 0.84$ , and  $2.22 \pm 0.55$ ,  $P=0.179$ ), and DCFH-DA ( $14.2 \pm 12.17$  and  $25.25 \pm 19.96$ ,  $P=0.197$ ) 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.

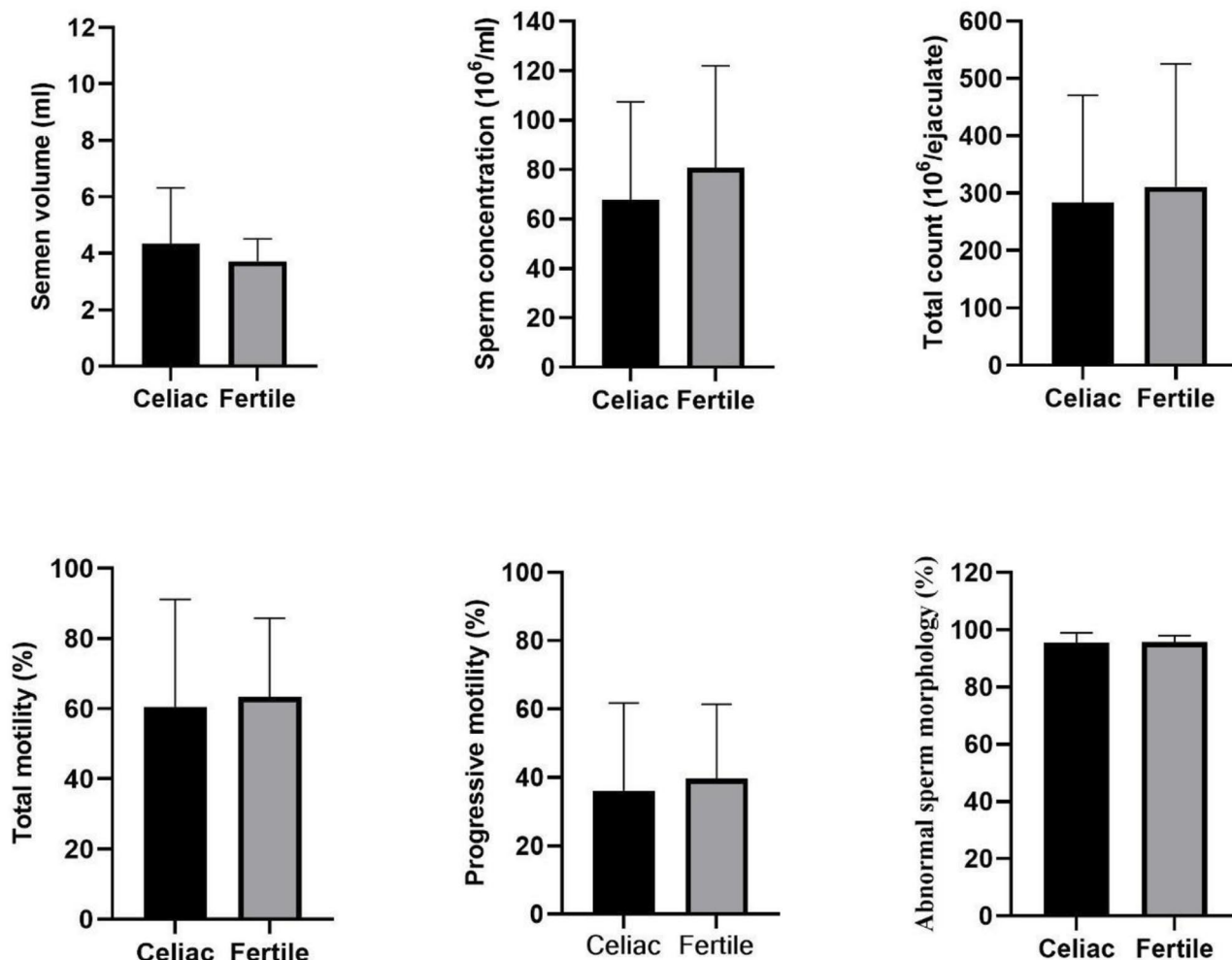
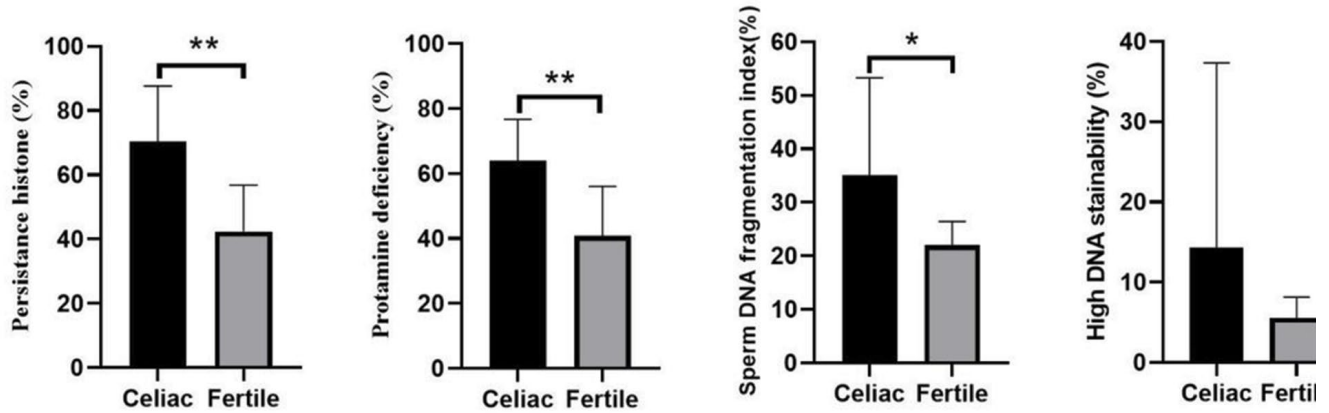
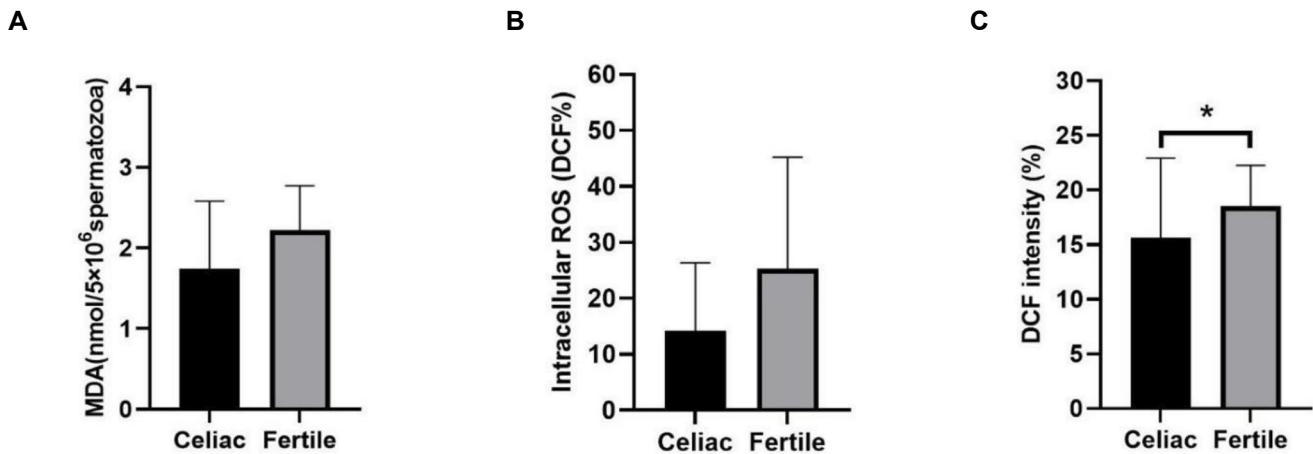


Fig.1: Comparison of semen parameters between 10 men with Celiac disease and 11 fertile men. Data are presented as mean  $\pm$  standard deviation (SD).



**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; Diacetylchlorofluorescein.

## 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 dihydrotestosterone, and raised serum luteinizing 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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