Cukurova Medical Journal
ÇUKUROVA ÜNİVERSİTESİ TIP FAKÜLTESİ

Cukurova Med J 2024;49(2):320-331
DOI: 10.17826/cumj.1415350

RESEARCH

The effects of exposure to microplastics in drinking water on sperm parameters and TNP1 and TNP2 sperm nuclear protein genes

İçme suyundaki mikroplastiklere maruz kalmanın sperm parametreleri ile TNP1 ve TNP2 sperm nüklear protein genleri üzerindeki etkileri

Harika Topal Önal[1], Tiinçe Aksak[1], İzzet Oğuz[2]

[1]Toros University, Mersin, Türkiye
[2]Mersin City Training and Research Hospital, Mersin, Türkiye

Abstract

Purpose: Microplastics have emerged as a significant concern amid the rise in environmental pollution. Nevertheless, the potential impacts of microplastics present in drinking water on human health remain inadequately understood. This study aims to explore the exposure to microplastics in drinking water among men who consume water from plastic bottles and carboys compared to those who consume tap water.
Materials and Methods: A total of 100 patients were included in the study, 50 of whom used only tap water and 50 of whom used plastic bottles and carboys. In addition, this study evaluated the effects of exposure on sperm parameters and potential effects on the expression levels of the sperm nuclear protein genes transition protein 1 gene (TNP1) and TNP2.
Results: According to the findings, it was observed that the incidence of amorphous headache anomalies among individuals using plastic bottled water was significantly higher compared to those using tap water. In addition, the coiled tail anomaly and the terminal droplet anomaly in the tail were also observed to be significantly higher in people using plastic bottles compared to those using tap water. The results show that exposure to microplastics in drinking water can affect male sperm parameters and alter the expression of TNP1 and TNP2 genes.
Conclusion: It was found that there were differences in exposure levels between those who consumed plastic bottles and carboy water, and those who consumed municipal water.
Keywords:. Microplastics, plastic bottle, sperm parameters, TNP1, TNP2, gene expression

Öz

Amaç: Mikroplastikler, çevresel kirlilik arttıkça önemli bir endişe kaynağı haline gelmiştir. Ancak, mikroplastiklerin içme suyundaki potansiyel etkileri hala yeterince anlaşılamamıştır. Bu çalışma, plastik şişe ve damacana su tüketen erkekler ile şebeke suyu tüketen erkekler arasındaki içme suyundaki mikroplastiklere maruziyeti araştırmayı amaçlamaktadır.
Gereç ve Yöntem: Çalışmaya toplamda 100 hasta dahil edildi, bunlardan 50'si sadece şebeke suyu kullanırken 50'si plastik şişe ve damacana suyu kullanmaktaydı. Ayrıca, bu çalışma maruziyetin sperm parametreleri üzerindeki etkisini ve sperm nüklear protein genlerinden Transition Protein 1 geni (TNP1) ve TNP2'nin ekspresyon düzeyleri üzerindeki potansiyel etkileri değerlendirdi.
Bulgular: Elde edilen sonuçlara göre, plastik şişe suyu kullananlarda amorf baş anomalisinin, şebeke suyu kullananlara göre belirgin şekilde daha yüksek olduğu saptandı. Ayrıca, plastik şişe kullananlarda bükülmüş kuyruk anomalisi ve kuyruktaki terminal damla anomalisinin de şebeke suyu kullananlara göre anlamlı olarak daha yüksek bulundu. Sonuçlar, içme suyundaki mikroplastiklere maruziyetin erkek sperm parametrelerini etkileyebileceğini ve TNP1 ile TNP2 genlerinin ekspresyonunu değiştirebileceğini göstermektedir.
Sonuç: Çalışma plastik şişe ve damacana su tüketenler ile şebeke suyu tüketenler arasında maruziyet düzeylerinde farklılıklar olduğunu ortaya koymuştur.

Anahtar kelimeler: Mikroplastik, plastik şişe, sperm parametreleri, TNP1, TNP2, gen ekspresyonu

Address for Correspondence: Harika Topal Önal, Medical Laboratory Techniques, Vocational School of Health Services, Toros University, Mersin, Turkey E-mail: harika.topal@toros.edu.tr
Received: 05.01.2024 Accepted: 11.04.2024

INTRODUCTION

The production, use, and release of various environmental pollutants as a result of industrialization have majorly contributed to damage in animal, human, and environmental health. Endocrine disrupting chemicals (EDCs) can interfere with endocrine functions and mimic the activities of endogenous steroid hormones through various mechanisms[1,2,3].

Microplastics are small plastic particles less than 5 mm in size that have become ubiquitous in various environmental compartments, including freshwater sources such as drinking water. These particles originate from diverse sources, such as plastic litter, microbeads found in personal care products, and the degradation of larger plastic debris. Microplastic contamination has been detected in drinking water sources worldwide, raising concerns about potential health impacts[3]. The potential impacts of exposure to microplastics on human health have raised concerns, prompting both in vivo and in vitro studies in this field. After ingestion through food and digestion, microplastics can have negative effects on the health of the organism. Considering the whole biological system, microplastics have been found to induce oxidative stress, inflammatory conditions, DNA breakage, chromosomal damage and genotoxicity within cells. They can also affect the immune system, leading to inflammatory effects and the development of neoplasia[4,5]. One area of concern is the reproductive system, as sperm parameters and gene expression may be vulnerable to environmental insults.

Recent studies have revealed that microplastics are extensively distributed in oceans, seas, drinking water, sea salt, and various products[6,7]. Polystyrene microplastics are particularly prevalent in the environment, making them a major component of environmental pollution. Polystyrene is a type of plastic commonly used in the manufacture of plastic-based products and packaging materials. In addition to polystyrene, other plastics such as polyethylene, polyethylene terephthalate, polyvinyl chloride and polypropylene are among the most commonly encountered plastics by humans. Numerous studies have investigated the effects of microplastics on the digestive system, as well as their cellular, chromosomal and cytotoxic effects[8,9].

The main plastic used in drinking water worldwide is polyethylene terephthalate (PET). In a study, water from 259 plastic bottles purchased in nine different countries and manufactured by 11 different companies was analysed using Red Nile Dye. An analysis of 259 bottles purchased from 9 different countries and manufactured by 11 different companies showed that 93% of these bottles contained approximately 10.4 microplastic particles larger than 100 microns per litre of water. The results showed that water in plastic bottles can contain varying amounts of microplastic particles, ranging from 0 to 10,000 particles per litre. It was also found that 95% of these particles were between 6.5 and 100 microns in diameter. These findings highlight the presence of microplastics in bottled water and the need for further research into their potential impact on human health and the environment. In comparison, tap water had about half the number of plastic particles larger than 100 microns (5.45 particles per litre) compared to bottled water[10,11]. It has been observed that 97% of microplastics in tap water are in the form of fibres, while in bottled water 65% of particles are in the form of fragments and only 13% are in the form of fibres. In addition, microplastic particles in the size range of 6.5 to 100 microns were found to be present in an average of 325 particles per litre of water. This indicates a significant presence of microplastics in the water and highlights the potential exposure to these particles through drinking water consumption. The findings highlight the importance of understanding the sources and potential impacts of microplastics in water and the need for effective strategies to reduce their presence and protect both human health and the environment[12,13,14].

The rise in infertility rates in recent years has focused attention on gametogenesis and gamete quality. Infertility, once perceived as a predominantly female problem, is now increasing in men and there is a significant decline in semen quality[15]. In roughly 40% of men with impaired spermatogenesis, the cause remains unknown despite extensive diagnostic evaluation[16]. In addition to transporting a single haploid nucleus to the egg, a growing body of research suggests that sperm contribute to embryo development and offspring health through a highly responsive epigenetic signature. This signature is sensitive to environmental factors, including endocrine disrupting chemicals (EDCs), as well as the lifestyle of the father[17]. Changes in DNA methylation and chromatin organisation can adversely affect fertilisation and the embryo. These changes may also affect potential transgenerational effects and

susceptibility to disease burden in the offspring. Therefore, epigenetic changes in gametes are a major concern in terms of reproductive processes and the health of future generations 12. Exposure to the plasticiser bisphenol A (BPA) can affect spermatogenesis both centrally and locally. BPA can modulate steroid biosynthesis and induce apoptosis in germ cells and Sertoli cells. It can also interfere with the first wave of spermatogenesis, affecting the process of sperm cell formation. The effects of BPA can also disrupt the formation of the barrier that protects the internal environment of the testes, which can affect its healthy functioning. In addition, the effects of BPA may extend to non-coding RNAs and sperm quality. The effects of BPA on spermatogenesis represent an important area of research investigating potential adverse effects on the male reproductive system[18,19,20]. Indeed, the route and duration of exposure to microplastics have shown different outcomes on spermatogenesis. A study comparing urinary BPA levels with semen parameters in humans reported that BPA exposure resulted in lower semen quality[21,22,23].

A study of men without infertility found that higher BPA exposure was associated with abnormalities in sperm tails[22,23]. Another study reported a negative association between BPA levels and sperm motility, morphology and concentration, and a positive association between antioxidant levels and semen quality. In addition, this study found a positive association between DNA damage and seminal plasma lipid peroxidation levels with the presence of BPA[23]. These findings suggest that BPA exposure may have negative effects on sperm parameters and may have implications for male reproductive health. A study investigating the relationship between urinary BPA/phthalate metabolites and sperm parameters found that men with lower fertility rates had higher exposure to EDCs compared to the general population[24].

Although more than 100 mRNA molecules have been identified in human sperm, the exact mechanism by which these mRNAs inhibit sperm protein synthesis is not fully understood [25]. Some studies have emphasised that the transcription of spermatozoa during fertilisation affects the formation of the embryo, since it carries the paternal genome[26,27].

H2BFWT, a member of the H2B histone family, is one of the H2B variants found in male gametes. H2BFWT, primarily expressed in the testes, plays a crucial role in facilitating the histone-protamine transition, regulating the epigenetic control of gene transcription, and modulating its interaction with telomeres[12]. It is known that during the histone-protamine transition in sperm chromatin, transitional nuclear proteins play a critical role. The Transition Protein 1 gene (TNP1) is located on chromosome 2 and colocalizes with Transition Protein 2 (TP2), which regulates the interaction between DNA repair mechanisms and the DNA-nucleosome core. The transition protein TP1 encoded by TNP1 plays a crucial role in the regulation of protamine P2 and ensures the complete maturation of protamine[28]. TNP2 is known to exhibit different expression patterns among mammals. TP2 is a DNA-condensing protein that plays a crucial role in initiating binding to sperm DNA[12].

Many studies in the literature have investigated the effects of microplastics on human health. However, there is a gap in research specifically investigating the effects of microplastics in drinking water on human sperm. The hypothesis of this study is that men who consume water from plastic bottles and water dispensers are more exposed to microplastics in drinking water and that this exposure adversely affects sperm parameters. The study will also look at whether there are differences in the expression levels of the TNP1 and TNP2 genes between people who drink water from plastic bottles and water dispensers compared to those who drink tap water. Confirmation of these hypotheses will improve our understanding of the presence of microplastics in drinking water and their potential effects on male reproductive health.

The study investigated the potential effects of microplastics on sperm parameters and sperm nuclear proteins (TNP1 and TNP2) in men consuming bottled and tap water. It aimed to determine the level of exposure to microplastics in drinking water among individuals who consumed tap water compared to those who consumed bottled and packaged water, and to assess how this exposure may affect sperm cells and genes.

MATERIALS AND METHODS

Sample

A total of 100 patients were included in this study, which was conducted in collaboration with Toros University Vocational School of Health Services and Mersin City Hospital Andrology Laboratory. These

patients were those who were sent from the Urology Outpatient Clinic to the Andrology Laboratory for semen analysis.

The survey, which was in line with the aim of the study, was carried out with the consent of the male participants who volunteered to take part in the study (approval number 21.10.2022/5 of the Ethics Committee for Scientific Research and Publication of Toros University). The questionnaire asked about age, smoking, alcohol and continuous drug use, chronic diseases and history of varicocele. They were also asked what type of drinking water they preferred (tap water only, plastic bottle or carboy). Those who had regularly used plastic bottled water for at least 5 years were included in the study. The number of people to be included in the study was calculated in advance using the power analysis method. As a result of the power analysis performed using the G*Power 3.0.10 program, a total of at least 100 samples for 2 groups was found to be sufficient with 85% power and 5% margin of error (N1:50 - N2:50). A total of 100 patients were included in the study, 50 using only tap water and 50 using plastic bottles and carboys. Although people with varicocele disease were excluded, 12 people who had previously undergone varicocele surgery were included in this study.

Procedure

Semen samples were processed and evaluated according to the World Health Organization (WHO) criteria of 2021. Samples liquefied at 37°C for 15 minutes were counted and evaluated at x200 magnification in the Makler chamber. Taking into account the concentrations determined by counting, small aliquots were taken from the samples for PCR, corresponding to 5x10^6 cells per vial. The remaining samples were analysed by an ART (Assisted Reproductive Therapy) certified specialist at the Routine Andrology Laboratory of Mersin City Training and Research Hospital. Sperm concentration (10^6/ml), total motility (%) and sperm morphology (% typical or ideal shape) were measured in the semen samples. The smear samples were stained with Diff-Quick (DiffPlus Fast). Sperm morphology was assessed according to Kruger's strict criteria. Spermatozoa were divided into two groups: those with normal morphology and those with abnormal morphology. Abnormally shaped spermatozoa were subdivided into head abnormalities, midbody abnormalities, tail abnormalities, cytoplasmic droplets and amorphous shapes. These subheadings were also subdivided into certain known anomalies. Spermatozoa with normal morphology and all other abnormalities were reported as percentages (%).

RNA isolation stage from sperm

When isolating RNA from sperm samples, the innuPREP RNA Mini Kit 2.0 (Catalog No. 845-KS-2040050) was employed, following the catalog protocol. To achieve a concentration of 5 x 10^6 cells, sperm samples were placed in 1.5 mL Eppendorf tubes containing 400 μL of Lysis Solution. The sperm cells were then disrupted by pipetting, and the mix was incubated at room temperature for 2 minutes. Afterward, Spin D Filter tubes were prepared with collection tubes placed underneath, and 400 µl of the homogenized sample was transferred to the filter tubes. The tubes were centrifuged at 11,000 x g for 2 minutes, and the filtered tube was discarded while keeping the collection tube intact. To the sample in the collection tube, 400 µl of 70% ethanol was added and mixed. A Spin R Filter tube was prepared and the sample was transferred to it, with a maximum volume of 700 µl. The tube was then centrifuged at 11,000 x g for 2 minutes. After emptying the collection tube, the filtered tube was transferred to a new collection tube. Next, 500 µl of Washing Solution HS was added to the filtered tubes, and they were centrifuged at 11,000 x g for 1 minute. The collection tubes were emptied, and the filtered tubes were transferred to new collection tubes. Subsequently, 700 µl of Washing Solution LS was added to the filtered tubes, and they were centrifuged at 11,000 x g for 1 minute. The collection tubes were emptied, and the filtered tubes were transferred to these new collection tubes. The tubes were centrifuged again at 11,000 x g for 3 minutes. The collection tubes were discarded, and the filtered tubes were transferred to 1,5 ml Eppendorf tubes with caps. Then, 30-80 µl of RNase-free Water was added to each sample and incubated at room temperature for 1 minute, followed by centrifugation at 11,000 x g for 1 minute. The obtained RNA samples were stored in RNase-free water at -80°C. When they were to be used in RT-PCR, the RNA was converted to cDNA.

cDNA synthesis

cDNA synthesis was carried out with the Thermo High Capacity cDNA Reverse Transcriptase Kit (Catalogue No: 4368814). Before starting the procedure, all reagents and RNA samples were thawed. After thawing, a brief spin was applied to the

samples and reagents to ensure proper mixing (Table 1).

Thermal cycler protocol

1. 25°C for 10 minutes
2. 37°C for 1200 minutes (20 hours)
3. 85°C for 5 minutes

Table 1. cDNA synthesis protocol

10✕ RT Buffer	2 ul
25✕ dNTP Mix (100 mM)	0,8 ul
10✕ RT Random Primers	2 ul
MultiScribe™ Reverse Transcriptase	1 ul
Nuclease-free H2O	7,2 ul
Total RNA	7 ul
Total Volume	20 ul

RT-PCR

Commercial kits (Sigma) were used to obtain cDNA samples for the determination of gene expression levels. The specific primer sequences for the analyzed genes, TNP1 and TNP2, as well as the housekeeping gene GAPDH, were provided in Table 2. For each sample tube, 10 µL of Power SYBR Green PCR Master Mix, 1 µL of primers, 4 µL of RNase-free water, and 4 µL of cDNA were added. These were then placed in a real-time PCR instrument and subjected to the PCR conditions specified in Table 3. In a multiwell plate, 16 µL of the mixture was prepared for each sample, and 4 µL of cDNA was added on top of the mixture before loading it into the instrument.

Table 2. The genes and their corresponding primer sequences mentioned

Gene Name	Primer Sequence
TNP1	F:5'GGCAGAACTTACCATGTCGA3' R:5'ATCGCCCCGTTTCCTACTTT3'
TNP2	F: 5'CACCCAGACTCACAGCCTT3' R:5'ACGATGGCTCTGTCTGCAA3'
GAPDH	F:5'CTGCACCACCAACTGCTT3' R: TTCTGGGTGGCAGTGATG3'

Table 3. PCR conditions for gene analysis

PCR Steps		Target Temperature (ºC)	Standby Time (sec.)	Fluorescent Reading
Denaturation		95	600	None
Amplification(45 cycles)	Denaturation	95	10	None
	Annealing	60	10	None
	Extension	72	15	Single
Melting Curve	Relaxation1	95	30	None
	Relaxation2	65	60	None
	Reading	97	0	Continuous
Cooling down		40	30	None

Statistical analysis

The quantity of cDNA derived from sperm in this study was individually determined using the comparative ΔCt method. The threshold cycle (Ct) signifies the cycle number at which the fluorescence curve is generated within the reaction above the threshold for qPCR. ΔCt was computed by subtracting the Ct values of the target cDNA from the Ct values of GAPDH cDNA, expressed as ΔCt = [(Ct Target cDNA) - (Ct GAPDH cDNA)]. Subsequently, ΔΔCt was calculated by subtracting the mean ΔCt of the control groups from the ΔCt of the case groups, where ΔΔCt = [(ΔCt cases - ΔCt controls. The fold change cut-off for cDNAs was determined using the 2-ΔΔCt)] equation. The data obtained from RT-PCR analysis was analyzed using GraphPad software (GraphPad Prism Software version 5) with repeated measures ANOVA test. Statistical Chi-Square, NPar, and Kruskal-Wallis analyses were applied, and parametric and nonparametric tests were performed according to the results. $P < 0.05$ significant groups were determined by ANOVA and t-test.

RESULTS

When sperm parameters of those who use plastic bottled drinking water and tap water are evaluated, Table 4 and Table 5 demonstrate that there was no notable distinction between the groups concerning sperm concentration and sperm. However, when the results were examined, it was observed that the group using plastic bottles had lower parameters in terms of concentration, motility, and normal morphology than the group using tap water. The relationship between drinking water use and sperm morphology was evaluated in Tables 6 and 7. According to the results, it was observed that amorphous head anomaly was significantly higher in those using plastic bottled water than in those using tap water. In addition, the coiled tail anomaly and the terminal droplet anomaly in the tail were also observed to be significantly higher in people using plastic bottles compared to those using tap water. Table 8 shows that the cytoplasmic droplet anomaly was significantly higher in the group using plastic bottled water than in the group using tap water.

Table 4. The connection between the consumption of drinking water type and sperm concentration.

Drinking water type/ sperm concentration	<15X10^6/ml	≥15X10^6/ml	Asymptotic Significance (2-sided)*	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Tap water (N)	4	46			
Plastic bottle (N)	1	47			
Total (N)	5	93			
Fisher's Exact Test			.186	.362	.194

Table 5. The connection between the consumption of drinking water type and total motility.

Drinking water type/ total motility	<%42	≥%42	Asymptotic Significance (2-sided)*	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Tap water (N)	7	43			
Plastic bottle (N)	5	43			
Total (N)	12	86			
Fisher's Exact Test			.590	.760	.409

Table 6. The connection between the consumption of drinking water type and abnormal sperm morphology. (*: significance)

Drinking water type/ head anomaly	Elonge	Pyriform	Mac.	Mic.	Round	Multiple parts	Vacuole	Pinhead	Free head	Another abnormality	Amorph
Kruskal-Wallis	.287	2.280	4.946	.724	.889	.444	.851	.443	.917	10.039	.965
Asymp. Sig.*	.592	.131	.026	.395	.346	.505	.356	.506	.338	.002*	.002*

Table 7. The connection between the consumption of drinking water type and abnormal sperm morphology. (*: significance)

	Neck anomaly				Tail anomaly						
Drinking water type/ neck and tail anomaly	**Bent**	**Asymm.**	**Thick**	**Thin**	**Coiled**	**Dag**	**Short**	**Hairpin**	**Multiple parts**	**Broken**	**Terminal droplet**
Asymp. Sig.	2.568	.143	.878	1.900	.003*	.526	.203	2.614	.117	.265	.000*
Kruskal-Wallis	.109	.705	.349	.168	.954	.468	.652	.106	.733	.607	1.000

Table 8. The relationship between drinking water usage and cytoplasmic droplets.

		Levene's Test for Equality of Variances				Significance			
		F	Sig.	t	df	One-Sided p	Two-Sided p	Mean Difference	Std. Error Difference
Cytoplasmic droplet	**Equal variances assumed**	2.962	.089	-1.771	95	.040	.080	-1.34426	.75907
	Equal variances not assumed			-1.746	75.381	.042	.085	-1.34426	.76993

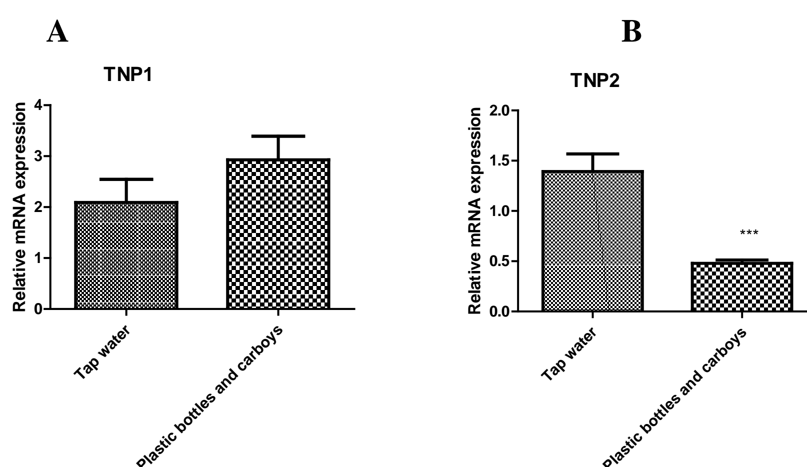

Figure 1. Comparative analysis of the relative expression profiles of TNP1 (A) and TNP2 (B) genes obtained from spermatozoa of individuals consuming tap water versus those consuming water from plastic bottles and water dispensers. The data derived from RT-PCR analysis were subjected to repeated measures ANOVA test using GraphPad software (***p<0.0001). TNP1 denotes the Transition Protein 1 gene, while TNP2 represents the Transition Protein 2 gene. The term "Case" refers to individuals consuming water from plastic bottles and water dispensers, whereas "Control" pertains to individuals consuming tap water.

The mRNA expression levels of the TNP1 gene were found to be increased in individuals using plastic bottles and tap water dispensers, in comparison to those using plastic bottles and carboys (Figure 1A). The expression level of the TNP2 gene in the plastic bottles and carboys group has shown a significant decrease compared to the control group. This difference was found to be highly statistically significant (***p<0.0001, case N:50, control N:50) (Figure 1B).

DISCUSSION

Lately, the widespread worry in society stems from the concerning impacts of environmental pollutants on reproductive health[29,30]. In recent times, the effects of environmental pollutants, particularly MPs have been attention because of their demonstrated capacity to induce a degree of toxicity within the gastrointestinal tract and liver of aquatic organisms and mammals. However, there remains a notable gap in our understanding concerning the precise impact of MPs on male reproductive processes in mammals.

H2BFWT, classified within the H2B histone family, represents one of the two distinct variants of H2B present in male gametes. The exclusive expression of H2BFWT in the testes underscores its fundamental roles: easing the transition between histones and protamines, orchestrating the epigenetic control of gene transcription, and governing its interplay with telomeres[45]. Within sperm chromatin, the pivotal involvement of transition nuclear proteins during the histone-protamine shift is well-established. Situated on chromosome 2, the TNP1 gene coexists with TP2, which governs the interrelationship between DNA

repair mechanisms and the DNA-nucleosome core[28]. The transition protein TP1, a product of the TNP1 gene, assumes a critical function in the regulation of protamine P2, thereby facilitating the comprehensive maturation of protamine [12].

Based on the findings of the study, individuals who utilize plastic bottles and water dispensers demonstrated a notable increase in mRNA expression levels of the TNP1 gene in comparison to those who utilize glass bottles and carboys (Figure 1A). Notably, the expression levels of the TNP2 gene were significantly lower among the cases than among the controls. This difference was found to be statistically significant (***$p<0.0001$, N=50 for cases and controls) (Figure 1B). There are opinions in the literature that reduction of the TNP2 gene may disrupt normal sperm function[12]. In our study, it was observed that TNP2 gene expression decreased, supporting this. This result suggested that the absence of TNP2 may cause infertility.

The factors contributing to the reduction in fertility rates over a lifetime are not yet fully comprehended. In addition to the natural decrease in the functioning of the reproductive system, aging brings about structural and operational changes in the testes. These alterations lead to a decline in semen quality characterized by shifts in sperm concentration and morphology, along with impairments in the development of sperm motility[31].

Several investigations have examined the quality of sperm in elderly individuals within both physiological and clinical contexts, as well as about lifestyle choices. A study by Paoli et al found that in terms of physiological changes, older men had significantly lower semen volume, progressive motility, and progressive motile sperm count than their younger counterparts. Simultaneously, there has been an increase in DNA fragmentation, coupled with a reduction in the expression of protamines (PRM1 and PRM2). However, the expression levels of TNP1 and TNP2 do not display the same decrease. Notably, the research also detected 67 microRNAs associated with pro-inflammatory states, mitochondrial functions, and NADPH oxidase complex activity, as well as the progression of spermatogenesis and depletion of stem cells. These microRNAs displayed distinct expression patterns in the seminal plasma of elderly subjects when compared to younger individuals. Hence, the significance of this research lies in its demonstration of age-related disruptions in the conducive microenvironment necessary for the molecular balance of sperm cells. Additionally, it sheds light on the emergence of genomic fragility during the natural aging process. Consequently, as individuals age, their sperm cells become more susceptible to accumulating damage from both internal and external factors, resulting in a decline in fertility rates[32].

Ming Zhao and their colleagues discovered that spermatogenesis in mice lacking the Tnp2 gene was nearly normal. In the study, it was observed that the weight of the testicles and the number of spermatozoa in the epididymis were not affected [33]. The anomaly observed was a minor elevation in sperm retention within stage IX to XI tubules. In Tnp2-null mice, epididymal sperm displayed an increase in abnormal tail morphology, with no significant changes in head morphology[33]. The researchers concluded that TP2 does not play a pivotal role in various aspects of sperm development. These aspects include shaping the sperm nucleus, displacing histones, initiating chromatin condensation, binding protamines to DNA, or impacting fertility. On the other hand, TP1 (Transition Protein 1) was found to be plenty expressed in all mammals studied. In mouse spermatid nuclei at stages 12 to 13, TP1 represented 60% of the basic proteins. TP2 levels, however, varied. Some suggested that TP2 first appeared during nuclear elongation, particularly around step 10 or 11, while TP1 appeared slightly later, around step 11 or 12. Initial studies in mice indicated that TP2 first appeared at step 12, peaked at step 13, and disappeared during step 14. However, a recent study reported the presence of TP2 as early as step 10[33].

Sperm collected from the epididymis of Tnp2-deficient (Tnp2−/−) mice exhibited a noticeable increase in tail abnormalities when compared to either wild-type or heterozygous mice (Table 2). Among the observed tail irregularities in Tnp2−/− mice, the most frequent types were sperm with midpieces that exhibited bending back on themselves (increasing from 5% in the wild type to 26%) and sperm in which the axoneme and/or outer dense fibers appeared unwound (increasing from 2% to 14%). In contrast, there was no elevation in the percentage of sperm displaying head irregularities, including those with blunt tips, a phenomenon previously noted in Tnp1-deficient (Tnp1−/−) mice[34]. This shows that TP2 is not crucial for spermiogenesis. The probable explanation is that the detrimental consequences arising from TP2's lack are

alleviated, first by TP1 and then through a compensatory mechanism by protamines.

Increasing infertility problems in recent years can be caused by the decrease in egg and sperm quality, especially in both men and women. Increasing infertility rates have caused researchers to focus especially on the gamete formation process. Although sperm parameters in men vary depending on the father's age, quality of life, smoking, and alcohol consumption, recent studies have shown that environmental factors, especially increased microplastics, may adversely affect male infertility. Unusual conditions experienced during the spermatogenesis process will affect embryo quality and adversely affect embryo development and quality[35]. In some studies, it has been reported that there is a decrease in sperm quality in groups exposed to environmental polluting agents.

Water, which is the source of life for humans, is a beverage that we have to consume constantly. Since childhood, individuals consume large amounts of water sold in plastic bottles as well as mains water. In particular, bisphenol A (BPA), a plasticizer found in pet bottles and plastic demijohns, can contaminate beverages as a result of exposure to sunlight[36]. Recent studies examining the effects of microplastics on water are noteworthy. Some studies have shown that exposure to BPA at different doses at different times affects the spermatogenesis process and hormonal regulation, and also causes the helper Sertoli cells to enter the apoptotic process. However, it has been reported that it disrupts blood-testicular barrier coordination, which is necessary for a healthy spermatogenesis process[37,38]. Studies in humans have been conducted by comparing BPA measurements in urine and sperm parameters, and it has been reported that high BPA levels reduce sperm quality. In one study, male mice were given MP with drinking water for 35 days and the number of viable sperm in the epididymis was examined in mice exposed to it. Mice exposed to MP showed a decrease in the number of viable sperm compared to the control group.

In addition, it has been reported that morphological anomalies and deformities are more common. In another study in mice, it was shown that MPs cause a decrease in sperm count and motility, as well as a decrease in morphological quality[36]. In our study, we examined the effects of MPs on sperm parameters in people with the habit of water in mains water and plastic pet bottles. When the spermatozoa morphologies were examined, especially in individuals using plastic bottles, it was found that the ratio of anomaly head, anomaly tail, and cytoplasmic droplet was statistically significantly higher than those using tap water. This result shows that MP accumulations in mammals can affect morphology during spermatogenesis, as seen in other studies examined. Although there was no notable difference of statistical significance between the two groups in parameters such as sperm concentration and total motility, it is noteworthy that these parameters were lower in people using plastic bottled water than in those using tap water. This suggests that the accumulation of MPs in tissues in higher mammals has not yet affected some parameters and that as their concentrations increase, they may adversely affect other parameters. However, it is noteworthy that the amorphous head anomaly is significantly higher in individuals who use only plastic bottled water. In spermatozoa, an amorphous head means a non-oval, elongated head. Anomaly in the head, where the genetic material is located, can cause serious problems in fertilization[39]. Studies show that chromatin and acrosome defects are high in amorphous-headed spermatozoa. Sperm with these defects cannot show acrosome reaction and have trouble passing through the zona pellucida layer of the oocyte. For this reason, especially in vitro fertilization trials, procedures performed with spermatozoa with amorphous heads give unsuccessful results in improved sperm parameters[6,40]. In a study conducted to investigate sperm with amorphous heads, neck, tail, and cytoplasmic droplet anomalies were observed more in an amorphous sperm compared to sperm with a normal head. This has also been shown as a reason for blocking fertilization. It is also known that amorphous-headed spermatozoa have less motility than normal sperm.

Motility is one of the important parameters affecting fertilization[41]. When the tail of a sperm with normal morphology suitable for fertilization is examined according to the Kruger criteria, it is expected to have a length of approximately 45 µm and a diameter of 0.5 µm. In addition, this part of the spermatozoa, which provides the movement, should not be curved or broken[42,43]. The significant increase in curved tails in the spermatozoa of the individuals in the group that was more exposed to MP observed in our study suggests that the movement rates of these sperms are limited. Studies show that mutations in the Dnah1 gene are associated with tail anomalies. This suggests that MPs may cause gene mutations. Future studies

may be informative in this area. Although the tail is broken and movement is restricted in patients who have undergone ICSI, spermatozoa with mutations should not be selected for the formation of a normal embryo. In addition, for natural fertilization to occur, the tail movement must be at a standard that can fertilize the oocyte. However, one of the results of our study was that the terminal droplets, another sperm tail anomaly, of those who use plastic bottled water, that is, those who are exposed to MP at higher rates, are significantly higher than those who use tap water. It is predicted that all these tail anomalies will lead to failure in motility-restricting, natural, and assisted reproductive techniques. The cytoplasmic droplet in spermatozoa can exist to a certain extent in the structure of mature sperm. However, droplets that are bigger than 1/3 of the sperm head are deemed abnormal.

This is an indication that the sperm has not been in the expected form for fertilization. The spermatozoa of the individuals in the group using plastic bottled water, which we observed similarly in our study, were found to have a significantly higher number of spermatozoa with cytoplasmic droplets compared to the other group [44]. This may be an indication that MPs can also affect the sperm maturation process and cause dysmaturations, which have not been mentioned so far and may shed light on future studies on this subject.

Considering all these results together, high dose and long-term exposure of MPs in humans, as in other mammals, may adversely affect the spermatogenesis process in male individuals, affecting sperm parameters and consequently impairing fertilization. Future studies in this area are important to reveal the effects of environmental pollutants on male fertility.

The findings of this study indicate that exposure to microplastics in drinking water could potentially have adverse effects on sperm quality and the expression of TNP1 and TNP2 genes. These findings are of significant concern as they indicate potential reproductive health risks associated with microplastic contamination. Further investigations are warranted to elucidate the underlying mechanisms by which microplastics affect sperm parameters and gene expression, as well as the long-term consequences on fertility and offspring health.

This research adds to our comprehension of the potential impacts of microplastics in drinking water on human health. The results highlight the need for more effective measures to protect drinking water sources. In addition, this study encourages more research into the relationship between microplastic pollution and reproductive health.

In conclusion, this study presents compelling evidence indicating that exposure to microplastics in drinking water could have detrimental effects on sperm parameters, encompassing count, motility, and morphology. Furthermore, it appears to disrupt the expression of TNP1 and TNP2 genes, which encode sperm nuclear proteins. These findings highlight the need for comprehensive assessments of microplastic pollution and its potential impacts on reproductive health. Efforts to mitigate and minimize microplastic contamination in drinking water sources are warranted to safeguard human fertility and overall well-being.

The limitations of our study include the limited sample size and the focus on a specific geographical region, as well as the examination of only the expression levels of the TNP1 and TNP2 genes, which are involved in sperm activity. In future studies, it is recommended to increase the sample size and collect samples from broader geographical areas to examine other genes involved in sperm parameters. This suggests the need for future research to conduct a more comprehensive and detailed analysis.

Author Contributions: Concept/Design : HTÖ, TA, İO; Data acquisition: HTÖ, TA, İO; Data analysis and interpretation: HTÖ, TA; Drafting manuscript: HTÖ, TA, İO; Critical revision of manuscript: HTÖ; Final approval and accountability: HTÖ, TA, İO; Technical or material support: İO; Supervision: HTÖ; Securing funding (if available): n/a.
Ethical Approval: The survey, which was aligned with the study's aim, was conducted with the consent of male participants who volunteered to join the study (approval No. 21.10.2022/5 from the Toros University Scientific Research and Publication Ethics Committee).
Peer-review: Externally peer-reviewed.
Conflict of Interest: Authors declared no conflict of interest.
Financial Disclosure: Authors declared no financial support

REFERENCES

1. Singh S, Li S. Epigenetic effects of environmental chemicals bisphenol A and phthalates. Epigenetics Pathol. 2013;13:267-78.
2. Ho SM, Cheong A, Adgent MA, Veevers J, Suen AA, Tam NN et al. Environmental factors, epigenetics, and developmental origin of reproductive disorders. Reprod Toxicol. 2017;68:85-104.
3. D'Angelo S, Scafuro M, Meccariello R. BPA and nutraceuticals, simultaneous effects on endocrine functions. Endocrine Metab Immune Disord Drug Targets. 2019;19:594-604.

4. Vethaak AD, Legler J. Microplastics and human health. Science. 2021;371:672-4.
5. Yee MSL, Hii LW, Looi CK, Lim WM, Wong SF, Kok YY et al. Impact of microplastics and nanoplastics on human health. Nanomaterials. 2021;11:496.
6. Smith M, Love DC, Rochman CM, Neff RA. Microplastics in seafood and the implications for human health. Curr Environ Health Rep. 2018;5:375-86.
7. Bhuyan MS. Effects of microplastics on fish and in human health. Front Environ Sci. 2022;10:82728.
8. Welle F, Franz R. Microplastic in bottled natural mineral water–literature review and considerations on exposure and risk assessment. Food Addit Contam Part A. 2018;35:2482-92.
9. Önal HT, Ayaz F. Effects of microplastics on the biological systems. Adv Eng Days (AED). 2022;4:36-8.
10. Oßmann BE, Sarau G, Holtmannspötter H, Pischetsrieder M, Christiansen SH, Dicke W. Small-sized microplastics and pigmented particles in bottled mineral water. Water Res. 2018;141:307-16.
11. Danopoulos E, Twiddy M, Rotchell JM. Microplastic contamination of drinking water: A systematic review. PLoS One. 2020;15:e0236838.
12. Amor H, Zeyad A, Hammadeh ME. Tobacco smoking and its impact on the expression level of sperm nuclear protein genes: H2BFWT, TNP1, TNP2, PRM1, and PRM2. Andrologia. 2021;53:e13964.
13. Doorn PF, Campbell PA, Amstutz HC. Metal versus polyethylene wear particles in total hip replacements: a review. Clin Orthop Relat Res (1976-2007). 1996;329:206-16.
14. Kim Y, Jeong J, Lee S, Choi I, Choi J. Identification of adverse outcome pathway related to high-density polyethylene microplastics exposure: Caenorhabditis elegans transcription factor RNAi screening and zebrafish study. J Hazard Mater. 2020;388:121725.
15. Andersson AM, Jørgensen N, Main KM, Toppari J, Meyts ERD, Leffers H et al. Adverse trends in male reproductive health: we may have reached a crucial 'tipping point'. Int J Androl. 2008;31:74-80.
16. Krausz C, Riera-Escamilla A. Genetics of male infertility. Nat Rev Urol. 2018;15:369-84.
17. Chianese R, Troisi J, Richards S, Scafuro M, Fasano S, Guida M et al. Bisphenol A in reproduction: epigenetic effects. Curr Med Chem. 2018;25:748-70.
18. Chianese R, Viggiano A, Urbanek K, Cappetta D, Troisi J, Scafuro M,et al. Chronic exposure to low doses of bisphenol A impacts the first round of spermatogenesis via SIRT1 modulation. Sci Rep. 2018;8:2961.
19. Li HM, Dai YW, Yu JY, Duan P, Ma XL, Dong WW et al. Comprehensive circRNA/miRNA/mRNA analysis reveals circRNAs protect against toxicity induced by BPA in GC-2 cells. Epigenomics. 2019;11:935-49.
20. Chioccarelli T, Manfrevola F, Migliaccio M, Altucci L, Porreca V, Fasano S et al. Fetal-perinatal exposure to bisphenol-A affects the quality of spermatozoa in adult mice. Int J Endocrinol. 2020;2750501
21. Radwan M, Wielgomas B, Dziewirska E, Radwan P, Kałużny P, Klimowska A et al. Urinary bisphenol A levels and male fertility. Am J Mens Health. 2018;12:2144-51.
22. Pollard SH, Cox KJ, Blackburn BE, Wilkins DG, Carrell DT, Stanford JB, Porucznik CA. Male exposure to bisphenol A (BPA) and semen quality in the Home Observation of Periconceptional Exposures (HOPE) cohort. Reprod Toxicol. 2019;90:82-7.
23. Omran GA, Gaber HD, Mostafa NAM, Abdel-Gaber RM, Salah EA. Potential hazards of bisphenol A exposure to semen quality and sperm DNA integrity among infertile men. Reprod Toxicol. 2018;81:188-95.
24. Caporossi L, Alteri A, Campo G, Paci E, Tranfo G, Capanna S, et al. Cross-sectional study on exposure to BPA and phthalates and semen parameters in men attending a fertility center. Int J Environ Res Public Health. 2020;17:489.
25. Güneş S, Kulaç T. The role of epigenetics in spermatogenesis. Turk J Urol. 2013;39:181-87.
26. Jodar M, Selvaraju S, Sendler E, Diamond MP, Krawetz SA, Network RM. The presence, role and clinical use of spermatozoal RNAs. Hum Reprod Update. 2013;19:604-24.
27. Sendler E, Johnson GD, Mao S, Goodrich RJ, Diamond MP, Hauser R, Krawetz SA. Stability, delivery, and functions of human sperm RNAs at fertilization. Nucleic Acids Res. 2013;41:4104-17.
28. Meistrich ML, Mohapatra B, Shirley CR, Zhao M. Roles of transition nuclear proteins in spermiogenesis. Chromosoma. 2003;111:483-88.
29. Inhorn MC, Patrizio P. Infertility around the globe: new thinking on gender, reproductive technologies and global movements in the 21st century. Hum Reprod Update. 2015;21:411-26.
30. Jin H, Ma T, Sha X, Liu Z, Zhou Y, Meng X et al. Polystyrene microplastics induced male reproductive toxicity in mice. J Hazard Mater. 2021;401:123430.
31. Almeida S, Rato L, Sousa M, Alves MG, Oliveira PF. Fertility and sperm quality in the aging male. Curr Pharm Des. 2017;23:4429-4437.
32. Paoli D, Pecora G, Pallotti F, Faja F, Pelloni M, Lenzi A, Lombardo F. Cytological and molecular aspects of the aging sperm. Hum Reprod. 2019;34:218-27.
33. Zhao M, Shirley CR, Yu YE, Mohapatra B, Zhang Y, Unni E, et al. Targeted disruption of the transition protein 2 gene affects sperm chromatin structure and reduces fertility in mice. Mol Cell Biol. 2001;21:7243-55.
34. Yu YE, Zhang Y, Unni E, Shirley CR, Deng JM, Russell LD, et al. Abnormal spermatogenesis and reduced fertility in transition nuclear protein 1-

deficient mice. Proc Natl Acad Sci USA. 2000;97:4683-88.

35. Amereh F, Babaei M, Eslami A, Fazelipour S, Rafiee M. The emerging risk of exposure to nano (micro) plastics on endocrine disturbance and reproductive toxicity: From a hypothetical scenario to a global public health challenge. Environ Pollut. 2020;261:114158.
36. Deng Y, Yan Z, Shen R, Huang Y, Ren H, Zhang Y. Enhanced reproductive toxicities induced by phthalates contaminated microplastics in male mice (Mus musculus). J Hazard Mater. 2021;406:124644.
37. D'Angelo S, Meccariello R. Microplastics: a threat for male fertility. Int J Environ Res Public Health. 2021;18:2392.
38. Lim CC, Lewis SE, Kennedy M, Donnelly ET, Thompson W. Human sperm morphology and in vitro fertilization: sperm tail defects are prognostic for fertilization failure. Andrologia. 1998;30:43-7.
39. Bianchi PG, Manicardi GC, Urner F, Campana A, Sakkas D. Chromatin packaging and morphology in ejaculated human spermatozoa: evidence of hidden anomalies in normal spermatozoa. MHR Basic Sci Reprod Med. 1996;2:139-44.
40. Hu M, Palić D. Micro-and nano-plastics activation of oxidative and inflammatory adverse outcome pathways. Redox Biol. 2020;37:101620.
41. Menkveld R. Clinical significance of the low normal sperm morphology value as proposed in the fifth edition of the WHO Laboratory Manual for the Examination and Processing of Human Semen. Asian J Androl. 2010;12:47.
42. Chemes HE, Sedo CA. Tales of the tail and sperm headaches to change concepts on the prognostic significance of sperm pathologies affecting the head, neck, and tail. Asian J Androl. 2012;14:14.
43. Nikolettos N, Küpker W, Demirel C, Schöpper B, Blasig C, Sturm R et al. Fertilization potential of spermatozoa with abnormal morphology. Hum Reprod. 1999;14:47-70.
44. Lehti MS, Sironen A. Formation and function of sperm tail structures in association with sperm motility defects. Biol Reprod. 2017;97:522-36.
45. Ding D, Pang MY, Deng M, Nguyen TT, Sun X, Xu Z et al. Testis-specific H2BFWT disrupts nucleosome integrity through reductions of DNA-histone interactions. bioRxiv. 2022; doi:10.1101/2022.07.20.500751.