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Original Article

Assisted reproductive treatments with hyperbaric oxygen therapy in male infertility

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ABSTRACT

Objective: This study aimed to analyze the results of hyperbaric oxygen therapy (HBOT) in addition to assisted reproductive technologies in male infertility cases.

Material and methods: Medical records of male infertility patients who had HBOT sessions for any reason between January 1, 2015-December 31, 2019, were analyzed retrospectively.

Results: A total of 15 male patients were included. Patients were classified as group 1 [DNA fragmentation (n=5)], group 2 [globozoospermia (n=5)], and group 3 [azoospermia (n=5)]. Round spermatid injection (ROSI), intrauterine insemination (IUI), or intracytoplasmic sperm injection (ICSI) procedures were performed in 10 of the 15 patients. A total of 31 embryos were obtained out of which 19 (61%) were transferred. While a total of 15 embryos were obtained in the globozoospermic group, which was the highest number, 10 were obtained from the azoospermic patients, and 6 from the DNA fragmentation patients (p=0.515). A total of 3 (30%) healthy pregnancies were obtained, overall. The mean sperm count of the patients (n=9) before HBOT was 8.4±11.1 mil/mL, while it was found to be 15.7±15.0 mil/mL after HBOT (azoospermic patients were not included). The TESE results were analyzed for azoospermic patients; positive changes were observed in 4 patients (80%). On the other hand, there was an improvement in 50% of group 1 according to sperm DNA fragmentation after HBOT (p=0.500).

Conclusion: While a total of 15 embryos were obtained in the globozoospermic group, which was the highest number, 10 were obtained from the azoospermic patients.. Further studies should be conducted on HBOT and male infertility.

Keywords: Assisted reproductive techniques; azoospermia; hyperbaric oxygenation; infertility; teratozoospermia.

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Introduction

Infertility is a significant social problem that causes social withdrawal and loss of self-confidence by affecting the mental health of couples. The female factor alone accounts for approximately 35% of infertility, both female and male factors for 20%, and the male factor is solely responsible for 30%.[1] Male infertility may have many reasons. In 90% of male infertility cases, there is a decrease in sperm count or the might show poor sperm quality.[1] Besides, sperm morphology disorders such as globozoospermia can lead to male infertility.[2]

Assisted reproductive technology (ART) is the most significant method of infertility treatment. Although many treatment methods have been developed, such as intracytoplasmic sperm injection (ICSI) and round spermatid injection (ROSI), the continued reality of the many couples who cannot have children motivates scholars to study possible solutions.

Hyperbaric oxygen therapy (HBOT) has been widely used in the treatment of many diseases. HBOT provides hyperoxygenation and resolves of edema. Also, HBOT, which is used as an effective treatment method in ischemia-reperfusion injury, has anti-inflammatory effects and stimulates the antioxidant system.[3-5]

We hypothesized that HBOT can improve the success of traditional infertility treatments. In

this study, we aimed to analyze the outcomes of HBOT application in addition to ARTs in male infertility cases, in terms of sperm count, testicular sperm extraction (TESE) results, sperm DNA fragmentation grade, number of embryos, and pregnancies. This study was planned as a pilot study.

Material and methods

The records of male infertility cases who declared to have had HBOT sessions for any reason between January 1, 2015-December 31, 2019, were analyzed. The inclusion criteria were (i) patients who had a history of infertility for at least one year, (ii) patients who did not have any urogenital tract abnormalities, (iii) patients whose spouses did not have any problems in terms of infertility evaluation, (iv) patients for whom live birth has never been achieved spontaneously or with ARTs at the first admission, (v) patients older than 18 years, and (vi) patients who had attended to HBOT 3 months before the ART processes' beginning. Patient data related to medical history, HBOT and ART applications, spermiograms, TESE results, available sperm DNA fragmentation analyses, and results of ART applications were gathered retrospectively from patient records. The main outcomes were classified as the sperm parameters (sperm count, TESE results, sperms DNA fragmentation rate), and the overall ART success parameters. Sperm parameters were compared between the results before HBOT and the results at least 3 months after HBOT. If a patient had undergone varicocelectomy before HBOT, sperm parameters were also compared before and after varicocelectomy, and after HBOT. The overall ART success parameters were defined as the number of embryos and pregnancies following the first ART cycle beginning a minimum of 3 months after HBOT (Figure 1). This study was approved by the Ethical Committee of Yuksek Ihtisas University Non-invasive Investigations (Approval number=2020/08/06, Date=21/08/2020).

The routine HBOT protocol is 100% oxygen at a pressure of 2.4 atmospheric absolute (ATA) for 120 minutes. Patients were also given routine antioxidant medical therapy (ZincTab 100 mg, Selenium 100 micrograms, CoEnzyme Q10 100 mg) for six months. Microsurgical varicocelectomy was performed on patients diagnosed with varicocele. Spermiogram test, Kruger criteria, and sperm DNA fragmentation test were applied to all patients. The TUNEL (TdT (terminal deoxynucleotidyl transferase)-mediated dUTP nick-end labeling) method was used. The most appropriate ART methods according to the results of the spermiogram or TESE was applied three months after the end of HBOT, provided that the patient reported having undergone HBOT. Spermatozoa, testicular spermatozoa, epididymis spermatozoa, and elongated spermatids were used for ICSI and ROSI techniques.

Micro-TESE was performed on non-obstructive azoospermic patients to look for all kinds of spermatogenic cells. During the process, seminiferous tubules were collected not only from the surface of the testicle but also from the depth of the testis, where normal-looking sperm were first searched for. TESE was applied by the same surgeon. As practiced in Tanaka et al.[6] hypo-os-

Main Points:

- This was a small sample sized pilot study, which was conducted to define a promising research area for HBOT and male infertility.
- The highest mean sperm count was found after HBOT.
- An improvement in the level of damage was observed in 50% of patients after HBOT application in the sperm DNA fragmentation patients.
- A total of 80% of our azoospermic patients showed improvement in the grade of elongated spermatid.
- After HBOT in 15 patients, 31 embryos were obtained as a result of IUI, ROSI, and ICSI methods, and 19 embryos were transferred. A total of 3 pregnancies were achieved.

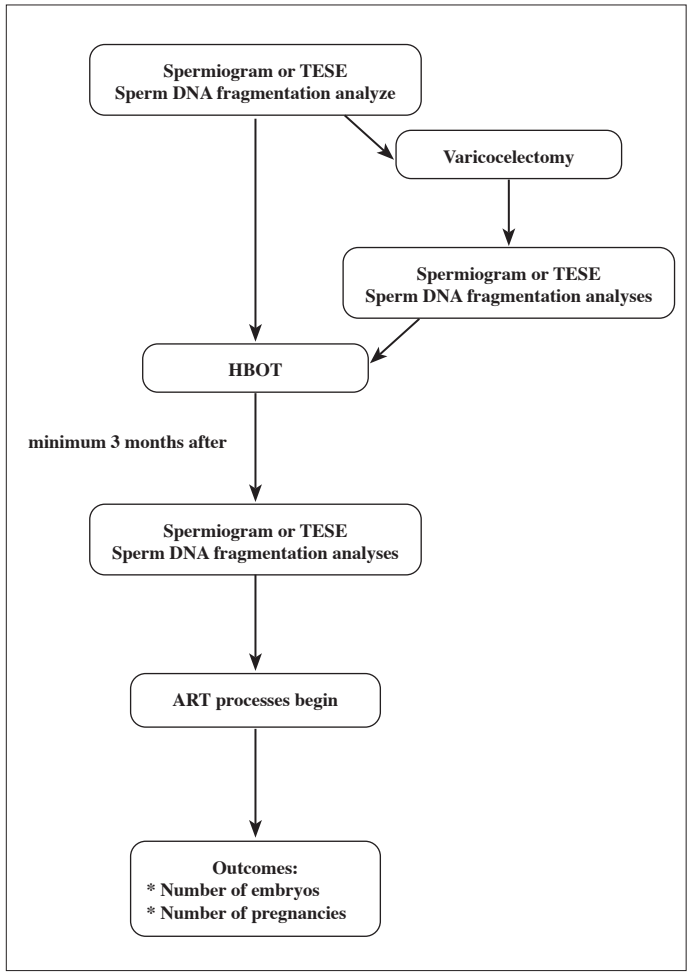

Figure 1. Flow Chart of the analyzed parameters
TESE: testicular sperm extraction; HBOT: hyperbaric oxygen therapy; ART: assisted reproductive technology

molality test, live/dead sperm staining tests were performed on immotile sperms, exposing them to 3.6mmol/L pentoxifylline to determine whether they were alive. In addition to the ROSI technique, the platinum piezoelectricity spermatid nucleus extraction method was applied to some of the patients. After the collection of testicular cells, round spermatids were identified as the first step, and the piezoelectric and nuclei extraction method was applied. During this process, ROSI procedures were performed starting from the injection of a spermatid into an egg in the same way that Tanaka et al.[6] applied. ROSI embryos were frozen before the transfer and pregnancy evaluation was performed 4-5 weeks after the frozen embryo transfer. In some cases, fresh embryo transfer was performed at the 4th and 5th days of embryo formation instead of embryo freezing depending on the condition of the embryo.

Statistical analysis

Data were analyzed using IBM Statistical Package for the Social Sciences (IBM SPSS Corp.; Armonk, NY, USA) version 25.0. Descriptive statistics were shown as frequency and percentage for categorical variables and mean, standard deviation, median, minimum, and maximum values for continuous variables. The difference between DNA fragmentation rates obtained before and after the treatment was evaluated by McNemar's test. The change in sperm count between the pre-treatment and the treatment process was evaluated with the Friedman test. The difference in the presence of pregnancy among the groups was assessed with Fisher's exact test. $P<0.05$ was considered statistically significant.

Results

A total of 15 male infertility patients were included in our study. The mean age of the patients was 35.4±6.9. The smoking data were available in 11 patients and 4 of them were active smokers. The mean length of marriage was 5.7±5.44 years. The median of the total number of HBOT sessions was 10 (8-10). None of the patients had given birth to a baby and only one patient had a miscarriage twice.

Sperm DNA fragmentation, globozoospermia, and azoospermia were detected as the main etiology for infertility. However, 11 of the patients (73%) also had a history of varicocele. All patients with varicocele (100%) had varicocelectomy before HBOT.

General statistics

Sperm count

In patients with varicocele, the results of the first spermiogram, spermiogram after varicocelectomy, and spermiogram 3 months after HBOT were compared (Table 1). There was no statistically significant difference between the median values (p=0.104). Data of azoospermic patients were not included in this analysis.

ROSI, IUI, and ICSI applications

According to the results of the spermiogram and TESE after HBOT, the most appropriate procedure was preferred among the IUI, ROSI, and ICSI applications. One of the ROSI, IUI, or ICSI procedures was performed in a total of 10 of 15 patients. ROSI was applied to 7 patients, IUI was applied to 2 patients, twice for each patient, and ICSI was applied to only 1 patient.

Number of embryos

As a result of these procedures, a total of 31 embryos were obtained and 19 (61%) of these were transferred. Since the number of embryos obtained as a result of IUI cannot be calculated, data of IUI were not included in the analysis of the number of embryos. The median number of embryos obtained via ROSI application was found to be 4 (Range = 0 - 8). Meanwhile, 3 embryos were obtained from 1 patient via a single ICSI application.

Number of pregnancies

A total of 3 (30%) healthy pregnancies were obtained in 10 patients as a result of IUI, ROSI, and ICSI procedures applied after HBOT. Twin pregnancy was obtained in 1 (14.3%) of the 7 patients who underwent ROSI. The pregnancy is currently in the 12th week without any problem. ICSI was applied to only 1 patient, who achieved 1 (100%) pregnancy, and she gave birth to a healthy baby. Pregnancy could not be obtained as a result of IUI in 2 patients, but 1 patient developed spontaneous pregnancy 2 months after IUI. That pregnancy is proceeding in a healthy way and is in the 24th week.

Analyses according to the etiology of infertility

For detailed analyses (n=15), patients were classified into 3 groups (group 1=DNA fragmentation, group 2=globozoospermia, and group 3=azoospermia) according to the etiology of infertility. The first group included 6 patients. As 1 patient had

Table 1. The comparison of the sperm count (mil/mL)

	N	Mean	SD	Median	Range	p
Spermiogram 1	9	8.4444	11.13442	5.0000	0.00-30.00	
Spermiogram 2 (after varicocelectomy)	9	12.9556	15.60233	5.0000	0.30-45.00	0.104
Spermiogram after HBOT	9	15.7778	15.03419	15.0000	0.70-45.00	

SD: standard deviation; HBOT: hyperbaric oxygen therapy

both globozoospermia and DNA fragmentation, the individual was included separately within the groups.

Sperm DNA fragmentation (group 1)

The sperm DNA fragmentation rates were compared between the first admission, after varicocelectomy, and a minimum of 3 months after HBOT. It was determined that in patients with >30% DNA fragmentation, the damage is high, in patients with 15%–30% DNA fragmentation, the damage is moderate, and patients with <15% DNA fragmentation are accepted as normal. Considering the extent of sperm DNA fragmentation, 66.7% (n=4) of patients had high damage, while 33.3% (n=2) had moderate damage. There was no change in the grade of DNA fragmentation after varicocelectomy. Varicocelectomy did not have a statistically significant improvement effect on sperm DNA fragmentation grade (p=1.000).

After HBOT, 1 (25%) of 4 patients with high damage regressed to normal, and 2 (50%) of the patients regressed to moderate level. Only 1 (25%) patient continued to have high damage. All of those with moderate damage (100%) also continued developing moderate damage. Overall, an improvement was observed in 50% (n=3) of patients with sperm DNA fragmentation after HBOT (Table 2). However, there was no statistically significant difference between DNA fragmentation levels before and after HBOT (p=0.500).

The sperm count of group 1 before, after varicocelectomy, and after HBOT is presented in Table 3. The highest sperm count was reached after HBOT. When the median sperm count values were compared, no statistically significant difference was found between these groups (p=0.331).

Globozoospermia (group 2)

Patients in group 2 (n=5) included globozoospermic patients. The detailed the sperm count of these patients is shown in Table 4. There was no statistically significant difference in sperm count (p=0.135).

Azoospermia (group 3)

In the control spermiogram of group 3 after HBOT, 2-3 elongated spermatids were detected in only 1 (20%) patient, and azoospermia was found in all the remaining patients. TESE results of these patients before and after HBOT are presented in Table 5. Accordingly, positive changes were observed in 4 patients (80%). While sperm could not be obtained in 1 patient in TESE

Table 2. Overall improvement in sperm DNA fragmentation rate after HBOT

		N	%	p
DNA fragmentation rate	No difference	3	50.0	
	Improvement after HBOT	3	50.0	0.500
	Total	6	100.0	

HBOT: hyperbaric oxygen therapy

Table 3. Comparison of sperm count (mil/mL) in Group 1

	N	Mean	SD	Median	Range	p
Spermiogram 1	6	12.1167	12.19515	6.0000	0.70-30.00	
Spermiogram 2 (after varicocelectomy)	6	18.5833	16.53607	16.5000	0.50-45.00	0.331
Spermiogram after HBOT	6	20.2833	16.34019	20.5000	0.70-45.00	

SD: standard deviation; HBOT: hyperbaric oxygen therapy

Table 4. Spermiogram results of patients in Group 2

Patient No	First admission	After varicocelectomy	After HBOT
1	3 mil/mL	4.3 mil/mL	4.4 mil/mL
2	0.7 mil/mL	0.5 mil/mL	0.7 mil/mL
3	1 motile, 2 immotile	Azoospermia	2 motile, 1 immotile
4	9-10 motile, 15-20 immotile	0.3 mil/mL	13-15 mil/mL
5	0.3 mil/mL	0.5 mil/mL	0.9 mil/mL

HBOT: hyperbaric oxygen therapy

Table 5. TESE results of azoospermic patients

Patient No.	Before HBOT	After HBOT
1	Grade 2 elongated spermatid	Grade 3 elongated spermatid
2	Sertoli Cell only	Sertoli Cell only
3	Grade 2 elongated spermatid	Grade 4 elongated spermatid
4	Grade 2 elongated spermatid	Grade 3 elongated spermatid
5	No cells found	Grade 3 elongated spermatid

HBOT: hyperbaric oxygen therapy

Table 6. Analysis of the number of embryos obtained by groups

Group	N	Mean	SD	Median	Min.	Max.	p
1	2	3.0	0	3	3	3	
2	3	5.0	2.6	4	3	8	0.515
3	3	3.3	3.1	4	0	6	
Total	8	3.9	2.4	3.5	0	8	

SD: standard deviation; Min: minimum; Max: maximum; HBOT: hyperbaric oxygen therapy

Table 7. The overall procedures performed, embryos obtained and transferred, pregnancies and fetuses by group

Group	ROSI	IUI	ICSI	Emb.	Transfer	Pregnancy	Fetus
1	1	0	1	6	6	1	1
2	4	4	0	15	8	1	1
3	3	0	0	10	5	1	2
Total	8	4	1	31	19	3	4

Emb.: embryo; ROSI: round spermatid injection; IUI: intrauterine insemination; ICSI: intracytoplasmic sperm injection

before HBOT, grade 3 elongated spermatid was obtained after HBOT. In 3 patients, however, elongated spermatid levels were found to be elevated after HBOT.

ROSI, ICSI, and IUI applications, number of embryo and pregnancies

The analysis of the procedures applied according to the groups was evaluated. The number of procedures, total number of embryos, transferred embryos, pregnancies, and fetuses in the groups are presented in Table 6. There was no statistically significant difference between the numbers of the embryo of the patients according to the groups (p=0.515). The highest number of embryos were found in globozoospermic patients.

In total, 1 pregnancy was obtained in each group, and no statistically significant difference was found between the groups in terms of pregnancies (p=1.000). Pregnancy, in group 2, was achieved spontaneously 2 months after the IUI procedure. The pregnancy in group 3 was a twin pregnancy. The overall analysis of ART procedures, the number of embryos obtained and transferred, and the number of pregnancies and fetuses by the group were analyzed and the results are shown in Table 7.

Discussion

In our study, we aimed to analyze the results of HBOT application in addition to ARTs in infertility cases. Although we could not define any statistically significant relationship, the highest mean sperm count was found in spermiograms obtained after HBOT. In the DNA fragmentation group, an improvement in the level of damage was observed in 50% of patients after the HBOT application. A total of 80% of our azoospermic patients showed improvement in the grade of elongated spermatid. After HBOT application in 15 patients, 31 embryos were obtained as a result of IUI, ROSI, and ICSI methods, and 19 embryos were transferred. A total of 3 pregnancies were achieved.

HBOT is an intervention involving the inhalation of 100% oxygen intermittently inside a hyperbaric chamber at a pressure higher than the pressure of the sea level (1 ATA).[7] HBOT pro-

vides hyperoxygenation in tissues and also reduces edema. It also inhibits neutrophil beta-2 integrin[3] and reduces inflammation by decreasing neutrophil adhesion to endothelium. It stimulates anti-inflammatory proteins.[4] It has been successfully used in ischemia-reperfusion injury, as HBOT promotes angiogenesis by increasing vascular endothelial growth factor production.[4] People may have doubts about oxidative stress. However, studies have shown that in routine HBOT protocols, the antioxidant defense system in our body is sufficient for ROS-related biochemical stresses to make it reversible.[4] HBOT triggers the response of the enzymatic antioxidant defense system.[5,8] Mitrović et al.[9] assumed that the energy-dependent mitochondrial activities and motility of the sperm could be improved by increasing oxygenation with HBOT. In other words, improvement in energy-dependent activities can positively affect the fertilization capacity of the sperm. Besides, as in varicocele, HBOT can be beneficial in cases where testicular circulation is impaired, or ischemia-reperfusion injury develops. The stimulating effect of HBOT on the antioxidant system and its anti-inflammatory effect can also be effective for sperm DNA damage caused by oxidative stress. In the literature, there are very few clinical studies on HBOT and male infertility.[9-11] Because of the lack of scientific data, we planned our study as a pilot study. We aim to direct our next research at observing the common effects of HBOT with other treatments in male patients with three different causes of infertility.

In this study, we evaluated sperm count analysis or TESE results. Since varicocelectomy was performed on 11 patients before HBOT, the effect of varicocelectomy was also assessed. The sperm count of the patients increased after varicocelectomy and HBOT. The highest mean sperm count was in spermiograms obtained after HBOT. However, there was no significant difference ($p>0.05$). The effects of varicocele on semen parameters and infertility are associated with increased testicular temperature, increased venous pressure, hormonal imbalance, epididymal dysfunction, autoimmunity, impaired acrosome reaction, renal-adrenal reflux, DNA damage, and oxidative stress. It has been reported that varicocele affects spermatogenesis by decreasing DNA polymerase activity through increasing temperature. Other studies indicate that chronic precapillary vasoconstriction in increased venous pressure, which is another mechanism, affects spermatogenesis by disrupting the circulation of the testicle.[12] In this respect, we believe that HBOT can provide hyperoxygenation in varicocele and stimulate angiogenesis, thereby improving testicular circulation.

Sperm count was re-evaluated according to the etiology of infertility. The increase in sperm count was not statistically significant in the sperm DNA fragmentation group and the globozoospermic group ($p=0.331$, $p=0.135$, respectively). In the azoospermic patients, TESE results were evaluated. Positive changes after HBOT were observed in 4 of 5 patients in this group. In the literature, there were 3 studies on the HBOT effect on sperm motility, sperm functional capacity, and sperm concentration. Mitrović et al.[9] applied HBOT (2.5 ATA, 90 minutes) on fresh sperm samples in oligospermic patients. A significant increase in sperm motor activity was reported 30 minutes after the end of HBOT ($p<0.05$). In another study, the effects of HBOT on sperm viability in rats with spinal cord injury (SCI) were investigated. A total of 7 sessions of HBOT (2.5 ATA) were applied to the study group (n=9); the first session was performed 12 hours after the SCI, one session completed per day. Sperm analyses were re-evaluated after 28 days. There was no significant difference in sperm concentration ($p=0.410$). A 2-fold increase in sperm viability was observed in the HBOT group compared with the control group ($p=0.001$). There was no decrease in sperm count in patients who developed SCI, but the sperm sample was mostly composed of dead sperms. The researchers stated that HBOT could benefit this group of patients by increasing sperm viability.[13] Zheng et al.[10] examined the effect of the combination of varicocelectomy and HBOT in patients with varicocele. It was stated that HBOT had positive effects on sperm quality, sperm penetration, and pregnancy rate. By providing a hyperoxygenated environment, HBOT can have a more positive effect on the energy-requiring functions of sperms rather than the number of sperms.[9] The increase in sperm motility, which has a significant role in fertilization, will also lead to an improvement in other fertilization parameters.[9,14]

Spermatogenesis and maturation can be affected by hormonal imbalances, temperature, diet, and exposure to toxins (cigarette, alcohol, cadmium, iron, radiation, and pesticides). In recent years, it has been understood that oxidative stress is a significant factor for sperm functions.[14] Excessive ROS production reduces sperm quality by decreasing sperm motility and viability.[13] It initiates an apoptotic cascade and causes DNA fragmentation in spermatozoa.[9] Sperm DNA damage causes apoptosis in spermatozoa, problems in embryo development, and pregnancy losses. Studies have shown that people with sperm DNA damage have higher infertility rates. It is believed that, by reducing the semen quality, DNA damage in spermatozoa leads to low preimplantation rates, higher risk of miscarriage, and an increased risk of childhood cancer.[14]

In our study, while there was no change in the degree of DNA fragmentation after varicocelectomy, an improvement in the level of damage was observed in 50% of patients (n=6) after the HBOT application. However, no statistically significant difference was found. In another study, the sperm DNA fragmentation rate decreased from 33.2±7.5% to 11.9±5.9% in the group where HBOT was applied.[11] No other studies analyzing sperm DNA fragmentation have been found in the literature. Metelev et al.[11] also analyzed ROS in semen. The median value of ROS

in semen decreased from 0.89 to 0.39 mV/s in the HBOT group ($p<0.05$). We believe that, by stimulating the antioxidant system and creating an anti-inflammatory effect, HBOT may have a positive effect in this group where oxidative stress is dominant.

New therapeutic advances in technology have increased the success rates of fertility in infertile patients. One of the causes of infertility examined in our study is globozoospermia, characterized by round-headed spermatozoa, in which the acrosome does not develop.[15] There was no treatment option for globozoospermic patients before ICSI. However, when compared with the general population, the success of ICSI application in globozoospermic patients is still low.[16] Another patient group included in our study was azoospermic patients. Sperm cannot be obtained in azoospermia, so ARTs cannot be applied.[17] Patients with azoospermia or elongated spermatids may also have round spermatids. Although the success rate is currently low, ROSI has at least enabled some azoospermic men to have their genetic children. One of the most critical problems with ROSI is that round spermatids obtained with MicroTESE from men with spermatogenic failure cannot fertilize an oocyte very well.[18]

In our study, after HBOT application in 15 patients, 31 embryos were obtained as a result of IUI, ROSI, and ICSI methods, and 19 embryos were transferred. A total of 3 pregnancies were achieved. Previously, only 1 patient developed 2 pregnancies and resulted in a miscarriage. None of the other patients had a pregnancy before the study. Metelev et al.[11] examined sperm samples of male infertility patients with sperm DNA fragmentation 3 months after the HBOT application. It was determined that the pregnancy rate was 63.3% (38/60) after IVF application in the HBOT group (n=60), while the pregnancy rate obtained with IVF in the control group (n=30) was 36.7% (11/30). This was reported to be statistically significant ($p<0.05$). In our study, 3 groups were formed according to the etiology of infertility. In total, 1 pregnancy was obtained in each group, and no statistically significant difference was found between the groups ($p=1.000$). There was no significant difference between the groups in terms of the number of embryos obtained ($p=0.515$).

The main limitation of this study is the small sample size. However, this study was designed as a pilot study that aimed to determine the infertility subgroup to be studied in further researches. Also, the absence of a homogeneous group and a control group lead to limitations for interpretation of results. Finally, the lack of details of previous failures in infertility treatments might also shade the results.

As a result, considering the patient groups, no difference was observed in terms of the number of pregnancies in our study. While a total of 50% of patients with sperm DNA damage (n=6) had lower grade after HBOT, 80% of azoospermic (n=5) patients showed improvement in the grade of elongated spermatid. The highest number of embryos reached in the globozoospermic patients was 15. A total of 10 embryos from azoospermic patients and 6 from DNA fragmentation patients were obtained. This can be considered a remarkable result for globozoospermic and azoospermic patients with very low treatment success. Further, the mean length of the marriage of our patients was 5.7±5.44 years, and it should be taken into consideration that there were patients who failed long-term infertility treatment. We hope that our study can give researchers an idea for future studies. We think that well-designed prospective controlled studies should be carried out on both *in vivo* and *in vitro* to reveal the effect of HBOT on sperms.

Ethics Committee Approval: Ethics committee approval was received for this study from the ethics committee of Yuksek Ihtisas University Non-invasive Investigations (Approval number=2020/08/06, Date=21/08/2020).

Informed Consent: Written informed consent was not obtained from who participated in this study due to retrospective design of the study.

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Conflict of Interest: The authors have no conflicts of interest to declare.

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