Investigation of the sperm aquaporin molecules expression in fertile and infertile men

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Abstract

Aim: Infertility is pregnancy failure for a year without protection, despite regular sexual intercourse. Various disorders and deficiencies in both genders can cause this situation, and a considerable portion of these cases consists of male infertility. We aimed to investigate the relationship between aquaporin molecules expressed in human sperm cells and male infertility.

Materials and Methods: We carried out the study on the sperm cells of two different groups consisting of ten fertile and twenty infertile men older than eighteen. Smear slides were prepared following spermiogram testing. These slides were stained using the Diff-quick and immunohistochemistry protocols to test the Kruger strict sperm morphology and detect the immune expression of the AQP molecules, respectively.

Results: The results of the spermiogram and Kruger-strict sperm morphology analysis showed that average sperm cell count and numerical sperm parameters values in the semen of infertile individuals decreased, and sperm counts with anomalies were higher in infertile ones versus those of fertile (p<0.05) as predicted. Our immunohistochemical analysis findings revealed that the immune expression of each aquaporin molecule was reduced slightly in infertile men regarding fertile ones, but the decrease was not statistically significant (p>0.05).

Conclusion: According to our findings, the immune expression of infertile ones' aquaporin molecules was reduced by a small amount, even if this was not statistically meaningful. These molecules of the sperm cells can not be primarily associated with male infertility. However, further advanced-level research is required on this issue to make a precise decision.

Introduction

Infertility is the inability to achieve pregnancy after one year despite regular and unprotected sexual intercourse, and this situation is increasing day after day [1]. Based on WHO statistics, a considerable portion of couples in developing nations experience primary or secondary infertility making it a growingly significant public health issue. Infertility is caused by male factors in 30% of infertile couples and by both male and female factors in 20% [1-3]. In the etiology of infertility cases, the direct and indirect effects of male factors are about 50% [1-5]. Moreover, in a recent study, Qi et al. reported that male factors are present in 20-70% of infertility cases [6].

Research on male infertility must also focus primarily on sperm cells that carry out fertilization, besides the other factors. Although some male infertility cases are caused by known reasons such as varicocele, undescended testes, infection, environmental factors, and genetic causes, up to 70% of them are idiopathic [3].

Spermatozoa proteins have an essential role in sperm morphology, motility, vitality, and in all physiologic events that enable sperm to achieve fertilization [7]. In this respect, male infertility may result from the impairment of some sperm molecule expression [8]. Both the viability and fertilization capacity of sperms may depend on many molecules, including aquaporins expressed in sperm cells. Aquaporin molecules (AQPs) are integral membrane proteins that act as channels that allow water and small solutes to pass through membranes [9, 10]. AQPs discovered in 1992 belong to membranes' major intrinsic protein (MIP) superfamily [11]. 13 different AQPs, from AQP-0 to

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AQP-12, are found in humans [2, 12]. Of these 13 isoforms of AQPs, 11 are expressed in the human reproductive system, and some of these like AQPs 3, 7, 8 are expressed in human sperm cells. These AQP molecules enable the transport of water molecules in the plasma membranes of sperm cells [13]. Their function is related to cell volume control in response to the osmotic changes encountered passing from the epididymal fluid to the cervical mucus and cytoplasm removal during sperm maturation [14, 15]. Therefore, they can directly or indirectly affect the vitality and movement of the sperm cells [12, 15, 16].

Since the incidences of male infertility have been progressively rising globally for various reasons, it is crucial to research the factors that can cause male infertility. AQPs' expression patterns may be related to paternal infertility due to their substantial roles in sperm health and function. In the literature, the available data are scant and inconsistent. Moreover, few studies directly handle the expression pattern of AQPs in paternal infertility. Therefore, in this study, we aimed to research the AQP molecule expression in sperm cells of fertile and infertile men to contribute to the literature about the correlation between AQPs and male infertility.

Materials and Methods

Study design

The study was carried out on semen samples taken from people who applied to the Andrology laboratory of Tokat Gaziosmanpaşa University. After the Human Experiments Clinical Research Ethics Committee was approved (No. 20170122), the study was started. Before conducting the research, a written informed consent form was obtained from all participants in the study. For semen samples, a total of 30 men aged over 18 years with 2-7 days of sexual abstinence were included in the study. Among these individuals, those addicted to alcohol or drugs, with active disease, and taking medication or hormone therapy were also excluded. Fertile men had no problem having a baby in the last two years, and there was no female factor problem for infertile men. The men were divided into two different groups as follows: group 1 (infertile), who have had a regular and unprotected sex life for at least one year and cannot have a child (n=20); group 2 (fertile), who do not have any reproductive and or medical problem (n=10). Sperm samples were given into sterile urinary dishes through masturbation by the men. After
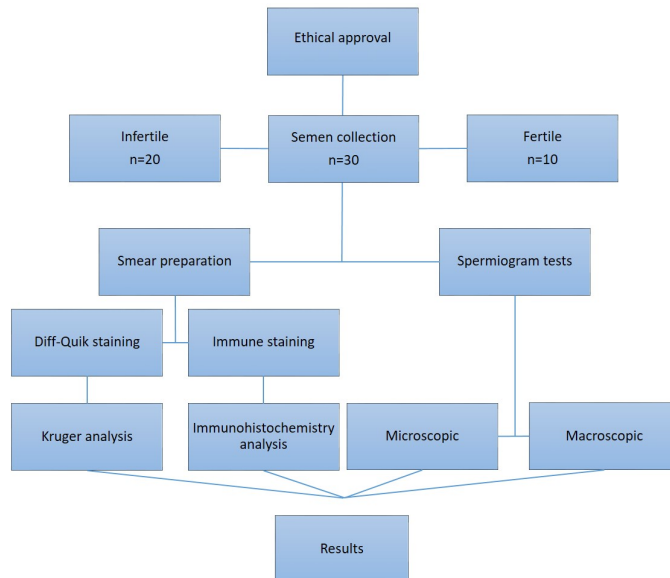

Figure 1. Flowchart of the study.

the collection of semen samples, sperm smear preparations, fixation of the sperm smears with 4% buffered neutral formalin, spermiogram test, Kruger's strict morphology, and immunohistochemical analyses were performed in the research laboratories of the Department of Histology and Embryology at Tokat Gaziosmanpaşa (Figure 1). All the analyses were conducted by a researcher blinded to the groups via a coding system.

Spermiogram test

Semen analyses were performed based on the WHO 2010 criteria, explained briefly in Table 1 [17]. After ejaculation, semen samples were evaluated in terms of liquefaction and viscosity within 30-60 minutes. Viscous samples were liquefied using α-chymotrypsin then the semen volume was measured. Each semen sample was microscopically evaluated regarding aggregation, agglutination, epithelial cells, and round cells such as leukocytes and immature germ cells. We performed the semen analyses using a Makler Counting Chamber. Briefly, a drop of the semen sample was dropped into the center at a depth of 10 microns of the Makler chamber and covered with a coverslip. Since only one sperm head can place at a depth of 10 microns, the sperm cells can be counted easily under a 20x objective. Sperm cells have been counted in 10 out of 100 squares, each 0.1-micron square. The results are expressed as million of sperm per milliliter.

Sperm smear preparation

Sperm smears were prepared to perform the Kruger morphology and immunohistochemical analyses. After having been mixed in one in five (1:5) ratio with phosphate buffer solution (PBS) in test tubes, the ejaculate was centrifuged, and the supernatant was removed. After washing, a small amount of the pellet diluted with PBS was dropped on the slide. The droplet was stretched on the slide surface using another clear slide, then dried at room temperature and fixed with 4% neutral buffered formalin.

Table 1. Minimum reference limits for human semen characteristics.

Parameter	Reference values
Abstinence time (days)	2-7
Volume (ml)	1.5
Viscosity	fluid/viscous
Liquefaction time (minute)	$\leq$60
Sperm concentration (per ml)	$\geq$15
Total sperm number (per ejaculate)	$\geq$40
Total live sperm (%)	>58
Total motility (PR + NP, %)	>40

Diff-Quik staining and Kruger analysis

Sperm diff-quick staining was conducted for Kruger's strict morphology analysis. The sperm smear slides were left sequentially for 10-15 seconds in each Diff-Quik stain kit, including fixative (methanol), solution-I (eosinophilic xanthene), and solution-II (basophilic thiazine) agents, followed by water rinse and dry. The diff-quick stained slides were mounted with a coverslip and analyzed under a microscope. Kruger's strict sperm morphologic analyses were performed based on the modified WHO-2010 criteria. In the criteria, some morphologic abnormal forms of human spermatozoa are as follows, briefly: Head defects: tapered, pyriform, round (no acrosome, small), amorphous, vacuolated, small acrosomal area. Neck and midpiece defects: Bent neck, asymmetrical, thick insertion, thin. Tail defects: Short, bent, coiled. Excess residual cytoplasm: Especially in the head, neck, and other regions [17]. Based on Kruger's strict sperm morphology analysis, a minimum of 200 sperm cells for each individual were analyzed through 100x objective under a research microscope. Both normal sperm cells and abnormal sperm cells with anomalies in the main regions (head, neck, middle piece, and tail) were separately recorded. Then total and regional anomalies values were calculated.

Immunohistochemistry staining

An indirect immunohistochemistry protocol was performed on the semen smears to define immune expressions and the immune location of AQP-3 (Thermo Fisher Scientific USA), AQP-7 (Proteintech USA), and AQP-8 (Abcam UK) molecules in sperm cells.

Briefly, the slides were washed in PBS and incubated for 15 minutes in 1% bovine serum albumin (BSA) for blocking. The BSA was removed without washing, and then primary antibodies of AQP-3 (1:100), AQP-7 (1:100), and AQP-8 (1:100) were dropped onto the samples and incubated at 4°C in a humidified and dark chamber overnight. Washing with PBS for 5 min at room temperature, the samples were incubated with biotinylated secondary antibody (Vector Laboratories, USA) at room temperature in a humidified dark environment for 30 min and again washed with PBS incubated with horseradish peroxidase (HRP)-labeled streptavidin secondary antibody (GE Healthcare UK) at room temperature for 20 minutes. Immune expressions were made visible by adding aminoethyl carbazole (AEC, ScyTek Laboratories Inc.) onto the samples after washing them with PBS. A few slides were simultaneously treated with PBS instead of the primary antibodies for the negative control. No immune staining occurred in sperm cells on these slides (Figure 3 NC-F and NC-IF). Following counterstaining with hematoxylin and washing with distilled water, the slides were mounted under a coverslip in an aqueous mount reagent (Invitrogen, Carlsbad, CA).

Immunohistochemical semiquantitative-score analysis

Immunostaining intensities of AQP-3, 7, and 8 were evaluated using the Nikon NIS-Element software (Hasp ID: 6648AA61; Nikon) through a Nikon Eclipse E200 microscope. In each slide, randomly selected five fields were searched at x1000 magnification to detect immune staining densities and locations of the AQPs in the sperm cells.

To determine expression levels of the AQP molecules, the immune staining density of these molecules was graded at four categorical levels. The criteria of the immunostaining scoring scale are as follows: no staining (-), slightly detectable staining (+), moderate staining (++), and intense staining (+++). After measuring the mean immunostaining grade of each participant, the results were converted to immune staining H-score values [18]. The H-score is a semiquantitative evaluation system that is calculated as the sum of the proportion of cells in each staining intensity category times the weighed intensity score as H-score= $\sum$ Pi (i+1) where i is the staining intensity score, and Pi is the percentage of stained cells. The average immunostaining scores of the groups were calculated then the data were compared statistically.

Statistical analysis

Statistical analyzes were performed with the IBM SPSS-22 Windows Statistics package program (SPSS, IBM Co., Somers, NY, US). After testing the normality (Kolmogorov-Smirnov), independent Samples and Mann-Whitney U tests were used for total sperm number and total motile sperm number, respectively. Sperm anomaly values were compared with the Independent Samples Test, and sperm percentage values were compared with Mann-Whitney-U Test. Immunohistochemistry analyses were compared using the Independent Samples Test. Through the use of the G*Power 3.1.9.4 program, the sample size was determined. All data were expressed as mean ± standard error mean (SEM). P-values less than 0.05 were considered significant.

Results

Spermiogram test results

Semen analysis results have been shown in Table 2 and Table 3 as parametric and nonparametric, respectively. As expected, the numerical values of microscopic semen parameters were significantly higher in fertile individuals than those in infertile ones (p<0.01) (Table 2). There was

Table 2. Average values of the spermiogram test's microscopic analysis results of the study groups (number x 10^6).

	Sperm count/ml	Total Sperm	Total Live Sperm	Motil Sperm (%)	Non-motil Sperm (%)
Infertile	29.00±5	77.25±18	48.15±12	60.15±5	39.85±5
Fertile	106.57±7	286.43±33	247.29±28	93.00±2	7.00±2
P <	0.05	0.05	0.05	0.05	0.05

Table 3. Average values of the spermiogram test's non-microscopic analysis results.

	Volume (ml)	Liquefaction time (min)	Viscosity
Infertile	2.62± 0.2	38.50±2	fluid/viscous
Fertile	2.26± 0.1	40.00±3	fluid
P <	0.05	0.05	0.05

Table 4. Sperm morphological analysis results of the groups (±: SEM).

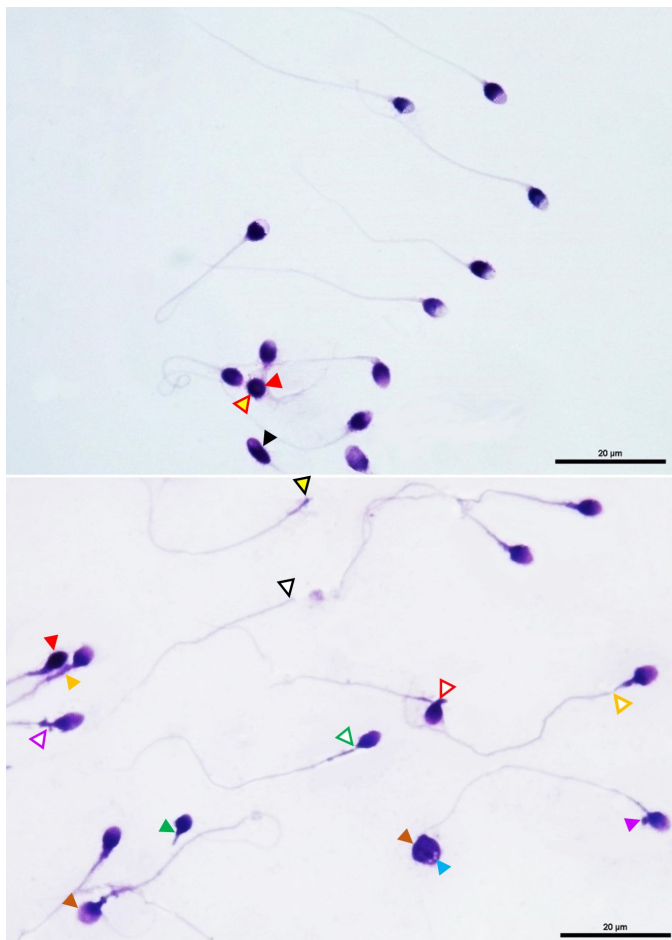|	Head	Neck	Tail	Total	Anomaly%	Normal%
			Sperm anomalies			
Infertile	20.95±1	66.05±2	73.40±2	160.4±3	79.80±1	20.20±1
Fertile	14.80±2	23.60±2	54.40±2	92.8±2	46.40±1	53.60±1
P <	0.005	0.005	0.005	0.005	0.005	0.005

Figure 2. Representative microscopic images of sperm cells from fertile and infertile groups. In the upper picture from the fertile group, mostly normal sperm cells are seen; in the lower image from the infertile group, there are many abnormal sperm cells. On the pictures, different colored arrows indicate some sperm anomalies; red: acrosomeless, red and yellow: round, black: elongated head, black and yellow: needle head, black and white: headless, yellow: thick neck, pink: cytoplasmic residue, pink and white: neck residue, white and red: bent neck, brown: large-headed, blue: acrosomal vacuole, white and green: thinned neck, white and yellow: abruptly thinned middle peace (Diff-Quik, 100xObj, Scale bar: 20µm).
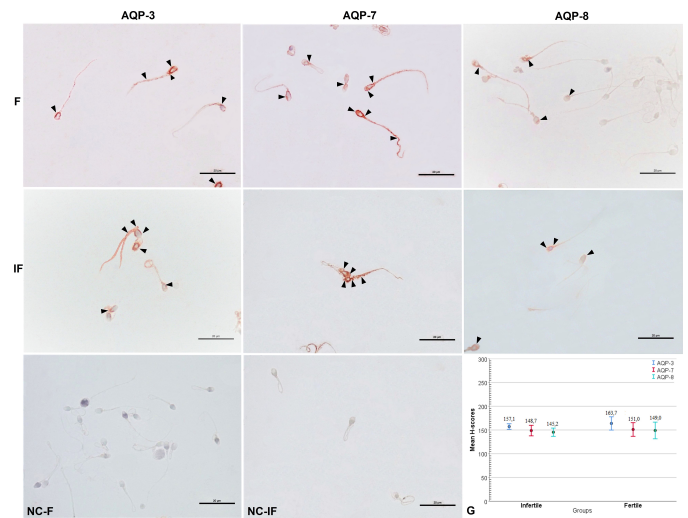

Figure 3. Representative photomicrograph of the immune-stained AQP molecules from the fertile and infertile group (F: fertile group, IF: infertile group, NC-F: negative control staining from the fertile group, NC-IF: negative control staining from the infertile group for AQP). Arrowheads indicate immune staining in some regions of the sperm cells. Image G: H-scores of the AQP molecules in the study groups (IHC, 100xObj, Scale bar: 20µm).

no difference between the groups in terms of spermiogram macroscopic parameters (p>0.05) (Table 3).

Kruger's strict sperm morphology analysis results

For Kruger's strict morphology analysis, Diff-Quik stained sperms were evaluated at the main regions of the sperm cells (the head, neck, middle piece, and tail) and as a whole. At the end of the analysis, it was observed that infertile individuals had a higher rate of abnormal sperm than fertile in terms of regional and total anomaly (p<0.05). The test results and a representative sample from each group have been shown in Table 4 and Figure 2, respectively.

Immunohistochemistry results

The mean immunostaining intensity H-scores and immune location of AQP molecules in the sperm cells of the study groups are given graphically in Figure 3 G. Representative microscopy images of AQP-3, AQP-7, and AQP-8 are shown in Figures 3 F and IF. The expressions of each AQP in sperm cells were not different between the groups regionally, like the head, neck, and tail. We observed that the immune expressions of AQP-3, 7, and 8 molecules in the whole part of the sperm were slightly higher in the control group. However, this increase was not meaningful when comparing the groups' mean immune staining H-score values for each AQP molecule (p>0.05).

Discussion

Male fertility is directly related to the availability of many physiologically and biologically active and quality sperm cells in terms of semen parameter criteria. Besides the

known, many factors that have not yet been detected may also play an active role in sperm fertilization capacity. According to World Health Organization (WHO) statistics, almost 50-80 million humans experience infertility worldwide [1, 13]. The direct or indirect contribution of male factors in the etiology of infertility cases is 50%, so we planned the study on this subject [4].

The routine semen analysis and evaluation of an individual semen quality may be necessary to detect male infertility. Therefore, we performed a spermiogram and sperm morphology analysis to determine whether our study groups consisted of infertile or fertile individuals. The study's fertile group's spermiogram microscopic test results, like sperm count/ml, total sperm count, and motile sperm percentage, significantly increased compared to the infertile group (Table 2). Moreover, in sperm morphological test analyses, we found that sperm counts with regional and total anomalies were significantly lower than those in the infertile group (Table 4). However, non-microscopical parameters such as volume, liquefaction, and viscosity were similar for each group (Table 3). These findings confirm that one study group is fertile and the other is infertile in this study.

Research on the causes and treatments for cases of infertility is also necessary. In this context, it is critical to study the pattern of expression and characterization of some molecules expressed in sperm cells that can impact the parameters governing the potential of sperm to fertilize an oocyte. Therefore we evaluated the expression characterization of AQPs 3, 7 and 8 molecules expressed in the sperm cell [15]. AQPs are water carrier proteins that allow the passage of water molecules through membranes, which are necessary for maintaining the vitality and functions of cells [13, 16]. The hypotonic environment is also important and can contribute to building up sperm morphology and motility. AQPs have essential roles in achieving this environment. Within this context, it is possible to claim that AQP molecules can improve sperm cell viability and fertilization abilities [19-23]. Since AQP 3, 7, and 8 molecules are localized in the plasma membranes of sperm cells, these molecules can be directly related to the vitality and movement of sperm cells [15].

Therefore, these molecules may affect the fertilization capacity of sperm cells and may be associated with male infertility. We observed this as a slight elevation of AQPs' immune expression in the fertile group.

Studies in the literature and our findings have shown that AQP-7 molecules are generally found around the head region and other parts; AQP-8 molecules are located mainly in the middle part; and AQP-3 molecules are usually seen in the human sperm tail plasmalemma [24, 25]. In the current research, the intense expression of AQP-8 molecules in the cell membrane of the middle piece of the sperm may also be caused by those in the membrane of the mitochondria. In terms of the localization of AQP molecules in sperm cells, no significant difference was observed between the groups, even though the results of our study are generally consistent with those of literature research.

In reaction to osmotic pressure, spermatozoa undergo physiological changes while they pass from the testicles to the female genital tracts. The maintenance of sperm cell size, shape, and volume is substantial for the function of the cell. Water and ion channels have an essential role in the arrangement of sperm volume. In a study, it has been revealed that AQP-8 has a critical role in the regulation of sperm volume, which is necessary for normal healthy spermatogenesis [13]. Another study concluded that AQP-7 and 8 contribute to the maturation of germ cells in testicles and play a role in spermatogenesis [26].

It has been reported that AQP-3 has a role in the physiologic hypotonic stress required for sperm motility and regulatory volume decrease, which is common in the sperm of infertile patients, inhibits sperm movement, and ensures the balance between cell swelling and tail bending. In some animal studies, it has been noted that AQP-3 is associated with sperm cell cryo-tolerance [19, 21]. Chen et al. (2011) suggested that AQP-3 existed in the principal part of the sperm tail membrane [25]. On the other hand, Laforenza et al. (2016) showed that AQP-3 was found in the sperm head, middle region, and tail. In another study, AQP molecules, especially AQP-3, play an essential role in regulating cell size and maintaining the detoxification of human sperm cells is reported as well [27]. Regarding expression localization of the AQP molecules, our observations are generally similar to those in the literature research results.

In light of the above literature knowledge, it can be stated that some AQP molecules are more or less active than others in different periods or stages of the sperm cells. We studied only the mature stage and the same period of the sperm cell. The AQP expression characterizations may also change through the female genital tract. Therefore, we might have found that although the AQP molecules' immune expression was slightly increased in the fertile group, there was no statistical difference between the groups.

There are scant studies on the relationship between AQP molecules expressed in sperm cells and male fertility. The available studies' findings are inconsistent and related to the expression and localization of AQP molecules. A significant amount of new research is needed in this area. Our study has some limitations: Samples were obtained from only one center. There were not too many cases. We used only spermiogram, Kruger, and immunohistochemical analyses but could not use other protein-detecting methods like western blotting and did not evaluate oxidative damage or DNA fragmentation.

Conclusion

As a result, we observed that immunohistochemical expressions of AQP-3, 7, and 8 molecules in sperm cells of fertile and infertile men showed a slight decrease in infertile individuals versus fertile ones but were not statistically significant. From the results, it can be stated that the immune expression of AQP molecules in sperm cells that have not yet gone through the genital tract may not directly relate to male infertility. However, further advanced-level research is required on this subject.

Ethical approval

This research was approved by the Clinical Research Ethics Committee of Sivas Cumhuriyet University for eth-

ical approval [No.20170122]. After the Human Experiments Clinical Research Ethics Committee was approved, the study was started.

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