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Original Research

Age-Related Changes in Sperm Morphology and Analysis of Multiple Sperm Defects

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Abstract

Background: Analysis of sperm morphology defects (amorphous heads, abnormal acrosome, etc.) is useful for estimating the efficiency of spermiogenesis and sperm maturation. An advanced paternal age (more than 40 years) is associated with decreasing sperm count and reduced motility; however, there is little information on the effect of aging relating to sperm morphological defects. Moreover, searching for stable combinations of certain morphological defects in the same sperm can be useful for better understanding spermiogenesis. The aim of the study was to investigate age-related changes in sperm morphology and the prevalence of certain combinations of sperm morphological defects in men from the general population. **Methods**: Sperm morphology was assessed in 1266 volunteers from the Russian urban general population in different age groups (18–19, 20–24, 25–29, 30–34, 35–40, and over 40 years old). Two hundred sperm were evaluated from each semen sample (about 250 thousand spermatozoa in total). Sperm defects were classified according to the WHO laboratory manual (WHO, 2010). The total percentage of each sperm defect and the frequency of different combinations of sperm morphological anomalies for each age group were counted. Additionally, a similar analysis was performed for the groups of normospermia and pathozoospermia. **Results**: The frequency of coiled and short sperm tails increased in men over 40 years old compared to younger subjects; however, aging did not affect the percentage of morphologically normal sperm. It was shown that the combination of a misshaped head (amorphous, pyriform, and elongated) with a postacrosomal vacuole, acrosome defect, excess residual cytoplasm, or any anomaly of the midpiece or tail in the same spermatozoon were not random combinations of independent solitary defects. The increased frequency of combinations of coiled tails with amorphous, elongated, or vacuolated heads was observed in men older than 40 years. Sperm morphological defects, such as severely deformed heads (pyriform, elongated, and round) were more common in men with pathozoospermia compared to normospermic subjects. **Conclusions**: An age-related impairment in sperm morphology was found. Stable combinations of head defects with anomalies in the acrosome, midpiece or tail suggest that these defects may be the result of a general violation in the morphogenetic mechanism.

Keywords: sperm morphological defects; aging; male fertility; population-based study

1. Introduction

Infertility is considered a serious disorder, which affects 15% of couples [1]. It is known that male fertility mainly depends on semen quality, such as sperm number, sperm motility, and sperm morphology as well as the chromatin integrity in spermatozoa [2,3].

Even an ejaculate from a healthy man is known to contain many sperm with different morphological defects (amorphous heads, pyriform heads, abnormal acrosome, coiled tail, etc.) alongside the sperm considered morphologically normal. It is known that these defects result from disturbances to spermiogenesis in the testes and sperm maturation [4–6]. Spermiogenesis is a complex process that includes acrosome formation, nuclear reshaping, chromatin condensation, and tail assembly [7,8]. As a result, the round haploid spermatids transform into differentiated spermatozoa of species-specific form, with tightly packed chromatin, to protect sperm DNA from negative external factors during the transport of sperm within the female reproductive tract. Any disturbances in this process can result in not only the appearance of different sperm morphological defects, but also in an impairment of the sperm DNA integrity. For example, round sperm heads without acrosome, which were observed in subjects with globozoospermia, result from impaired acrosome morphogenesis and/or a disruption in the interaction between the acrosome and the sperm nuclear envelope [9]. Globozoospermic subjects have increased sperm DNA fragmentation [10] as well as abnormal chromatin packaging and protamine deficiency [11]. Moreover, several morphological defects, such as excess residual cytoplasm and coiling tail, may be caused by impaired sperm maturation in the epididymis [12]. To conclude, the percentages of sperm morphological abnormalities in the ejaculate (sperm morphology pattern) are considered to be specific for certain men [13] and characterize, to some extent, the spermiogenesis state in the testes and sperm maturation in the epididymis [2].

However, according to the criteria provided by the "WHO laboratory manual for the examination and processing of human semen", an assessment of sperm morphol-

ogy is often limited to counting sperm with normal morphology [3]. Numerous investigations revealed no relationships between the percentage of morphologically normal sperm and the probability of success in natural conception [14], intrauterine insemination [15], and fertilization *in vitro* [16]. It is considered by several authors that the morphology of sperm has minimal significance on the male reproductive assessment due to the lack of standardization during the preparation as well as reading the smears, uniform classification, and the subjective nature of morphological assessments [17,18]. However, by only counting the proportion of normal spermatozoa, we may lose the information provided by the diversity of sperm morphological defects in the sample. The relationships were revealed between the proportions of several morphological defects and male fertility [19], sperm DNA fragmentation [20], oxidative stress levels in sperm [21], sperm chromosomal aberrations [22], sperm chromatin condensation [23], delayed embryo development in the early stages, and decreased implantation rate in (in vitro fertilization) IVF programs [24]. Additionally, an increased incidence of elongated and pyriform heads was observed in the subjects with testicular hyperthermia. The percentage of spermatozoa with elongated heads was also increased in infertile men with varicocele, and urogenital infections, and in the case of workers in reinforced plastic production [25]. The percentage of elongated heads in men with varicocele decreased after embolization [26], thereby suggesting reversibility in poor sperm morphologies as a result of the treatment. These findings suggest that spermiogenesis is sensitive to external factors and that the incidences of sperm morphological defects are influenced by environmental factors, lifestyle, and diseases; thus, spermiogenesis impairment is reversible, thereby enabling therapy of teratozoospermia. To conclude, the frequencies of sperm morphological defects in the ejaculate are related to negative conditions affecting spermiogenesis and epididymal sperm maturation, which reflect the extent of chromatin compaction and DNA integrity as well as suggesting possible treatments for the patient. Therefore, the careful estimation of all sperm morphological abnormalities in the ejaculate is more informative in male reproductive assessments, the clinical management of infertile couples, and their choice of assisted reproductive technologies (ART) [27] than simply counting sperm with normal morphologies. Future research is necessary to identify the specific morphological defects impacting ART success [16] and to reveal the relationships between sperm morphology and reproductive status. Moreover, humans are characterized by extremely high proportions and diversities of abnormal sperm in comparison with other animal species [6]. Therefore, estimating the incidences of sperm morphological defects in men is useful for understanding the mechanisms of spermiogenesis and the factors affecting them.

However, to elevate the usefulness of sperm morphological assessments, it is necessary to better understand the mechanisms underlying sperm defects in morphogenesis and to attain standardization in sperm morphological assessments. It should be noted that the introduction of computerized vision and technologies using artificial intelligence can significantly simplify a very time-consuming task of sperm morphological estimation and help to standardize the assessment of sperm morphology [28]. Moreover, large-scale population studies are necessary to investigate the impact of environmental and genetic factors on spermiogenesis and sperm morphology patterns [29], to identify the relationship between sperm morphology patterns and different male reproductive disorders. Numerous population studies performed in recent decades have demonstrated severe regional and ethnic differences in sperm count, sperm motility, and the percentage of morphologically normal sperm alongside a decline in semen quality [30–35]. However, population studies on sperm morphology abnormalities are rare [19,29] and there is only a single population study, which included the analysis of sperm morphology patterns in men from Russia [36].

Population variability in sperm morphology is caused by an interaction of numerous genetic, environmental, and lifestyle factors. Age is known to be an important factor that affects the male reproductive system. The investigation of age-related changes in semen quality is very important due to late paternity, which are observed worldwide but especially in Western countries [37]. To date, the threshold for advanced paternal age has not been established but risks of infertility, poor ART outcome, and genetic abnormalities in the offspring are believed to increase in men older than 40 years [38]. A number of studies have shown that men over 40 years of age are characterized by a reduction in sperm concentration, motility [39,40], and percentage of sperm with normal morphology [10], whereas the sperm DNA fragmentation [41] and the semen level of oxidative stress are increased [42]. These disturbances in semen quality are probably the result of age-related changes: the impaired function of the reproductive accessory glands, the decreased capacity to repair cellular and tissue damage, decreasing germ cell numbers, lowered androgen levels, structural changes in the seminiferous tubules, vascular insufficiency, and the systemic age-related diseases [37]. However, to date, age-related changes in the sperm morphology pattern have not been revealed. Therefore, it would be interesting to study the effects of age on the frequencies of sperm morphological abnormalities in the general population, considering the usefulness of careful estimations of sperm morphology patterns for male reproductive function.

It is well known that a combination of several sperm defects can be observed in one abnormal spermatozoon (for example, pyriform head and excess residual cytoplasm, round head, and abnormal acrosome, etc.). However, it should be noted that some combinations of morphological defects appear more often than others. For example, neck defects were significantly more frequent in round-headed spermatozoa than in spermatozoa with normal heads [43]. Midpiece and tail defects as well as excess residual cyto-

plasm (ERC) were more frequent in sperm with pyriform heads compared to sperm with normal heads. The authors speculated that these abnormalities are the result of the same abnormal processes of spermiogenesis [13,43]. Therefore, these combinations may not be random, since the disturbances in certain processes of spermiogenesis can lead to disruption of the formation of several morphological structures in the sperm simultaneously. The analysis of possible defect combinations in the same spermatozoon could be useful for understanding spermiogenesis in humans. However, such studies have not been conducted before, especially in large population samples.

This study aimed to investigate age-related changes in sperm morphology and to analyze the patterns and prevalence of multiple sperm morphological defects in subjects from the general population. To achieve this goal, firstly, we studied the influence of age on the total frequencies of sperm morphological defects; secondly, we compared the observed frequencies of multiple morphological defects in the population with predicted frequencies to verify the hypothesis on non-randomness of the appearance of combined morphological abnormalities in sperm, and to reveal non-random combinations. Finally, the frequencies of non-random multiple sperm morphological defects were compared in men belonging to different age groups, as well as the subjects with normal and impaired sperm parameters (according to WHO reference value).

2. Materials and Methods

2.1 Study Population and Semen Analysis

Male volunteers (n = 1266) from the general population of people living in the cities of Arkhangelsk (n = 88), Novosibirsk (n = 387), Kemerovo (n = 256), Ulan-Ude (n = 293), and Yakutsk (n = 242) were enrolled in the study. We used the standardized recruitment protocol, which has previously been described in more detail [35]. Briefly, men were informed about the study through an advertisement on the Internet and at universities. Subjects without acute general or chronic diseases in an acute phase, genital tract infections, and those not taking medicines were included in the sample. All volunteers provided informed consent for participation in the examination and filled in a standardized questionnaire, which included information on their current age, place of birth, family status, previous or current urological diseases, and a history of fertility.

The semen samples were obtained by masturbation, and analyzed for sperm count, concentration, and motility, according to the 5th edition of the "WHO laboratory manual for the examination and processing of human semen" [3]. To investigate the frequencies and the patterns of morphological defect combinations of spermatozoa in men with normal and impaired spermatogenesis, the subjects were divided into two groups, according to the reference values for semen parameters provided by the "WHO laboratory manual…" [3]: normospermia (sperm concentration $\geq 15 \times 10^6$/mL, progressive sperm motility $\geq$32%, and percentage sperm with normal morphology $\geq$4%; n = 708) and pathozoospermia (sperm concentration $< 15 \times 10^6$/mL and/or progressive sperm motility <32 percentage, and/or percentage sperm with normal morphology <4%; n = 550), 8 subjects were not assessed and excluded from the analysis.

2.2 Sperm Morphology Analysis

To assess sperm morphology, ejaculate smears were prepared, fixed by methanol (for 1 min), and stained using a Diff-Quick kit (Abris Plus, Saint-Petersburg, Russia), according to the manufacturer's manual [3]. Spermatozoa morphologies were examined using the bright-field optical microscope (Axio Skop.A1, Carl Zeiss, Germany) at $\times$1000 magnification, with oil immersion. The microscope was equipped with a digital camera AxioScope and special software (AxioVision 9.0, Carl Zeiss, Oberkochen, Germany) to enable sperm morphometry to be performed. Head, midpiece, and tail measurements of the spermatozoa were performed, if necessary. Two hundred spermatozoa for each semen sample were assessed twice in random and blinded orders by a single experienced researcher (Maxim Kleshchev).

2.3 Sperm Morphology Classification

The sperm were classified as normal, according to the criteria for normal sperm morphology provided by the "WHO laboratory manual for the examination and processing of human semen" [3]. Other sperm were referred to as abnormal, including borderline forms. The classification of morphological defects was performed, according to the classification scheme proposed by this manual. Unfortunately, the manual does not provide exact definitions of each abnormal sperm morphology. Consequently, the additional criteria for definitions of each sperm defect category were used to standardize sperm morphological classifications in our study [44].

A total of 14 types of anomaly were considered.

1. *Amorphous heads*: Heads vastly misshaped, with irregular edges, asymmetric, with an expanded postacrosomal zone, although they are not referred to as pear-shaped or elongated heads. Amorphous heads are the most common anomaly in sperm samples. This defect is associated with an elevated DNA fragmentation index [45] and abnormal chromatin condensation [46].

2. *Pyriform heads*: Heads with an elongated and narrowed postacrosomal zone. The ratio of head length/width was $\geq$2, with the width being measured at the equatorial zone [44].

3. *Elongated heads*: Heads with a length/width ratio >2 [44], although unlike the pyriform heads, the widths of the acrosome and postacrosomal zones are the same. Pyriform and elongated heads are known to be common sperm defects caused by impaired spermiogenesis [13,47]. An increase in the percentage of elongated heads was observed in

the subjects suffering from varicocele [26] and associated with ultrastructural nuclear defects and aneuploidies [25].

4. *Round heads*: Heads with a length/width ratio = 1–1.2 [44]. The percentage of round-headed spermatozoa in subfertile men was higher compared to fertile men [43].

5. *Acrosome defects*: The acrosome is less than 40% or greater than 70% of the head area [3]. This type of defect mainly includes acrosomeless spermatozoa, which are unable to penetrate the zona pellucida, and the increased frequency of this defect is associated with impaired fertility [48] and increased DNA fragmentation [36].

6. *Vacuolated heads*: Heads with more than two vacuoles, which occupy more than 20% of the head area or are located in the postacrosomal region [3]. A vacuolated head is known to be a marker of impaired chromatin condensation [49].

7. *Thick midpiece*: A width of more than 1 µm [3].

8. *Thin midpiece*: A width of less than the principal piece width [44].

9. *Bent neck*: The midpiece forms an angle of 90º (or less) with the long axis of the sperm head [44].

10. *Excessive residual cytoplasm* (ERC): The large (more than 1/3 of the head area) cytoplasmic remnant is presented [3]. An ERC produces pathological amounts of reactive oxygen species (ROS) and affects sperm motility and fertilization potential [50].

11. *Asymmetrical neck insertion*: The attachment of the midpiece is not aligned with the central axis of the head [3].

12. *Double tail*: The spermatozoon has two or more tails.

13. *Coiled tail*: The tail is coiled itself (>360°) [3]. Coiled tails and an ERC are markers of impaired sperm maturation in the epididymis [3,51].

14. *Short tail*: A tail length of less than 45 µm [3].

Several examples of sperm morphology defects are presented in Fig. 1.

Morphologies of the head, midpiece, and tail were evaluated and each revealed a morphological anomaly for each spermatozoon, which was noted in the special MS Excel file. In total, 248,152 spermatozoa were evaluated. Then, these data were transferred to the database "Reproductive Potential Male Population of Russia" (https://www.sysbio.ru/rpm/), described earlier [52]. The database contains information on the morphological defects revealed in each spermatozoon. The web interface of the database allows for the selection of the spermatozoa with specific combinations of morphological abnormalities, to determine the frequency of these combinations, while simple filters can be implemented, such as city, nationality groups, sperm status, and the age of the volunteers.

The percentages of each sperm defect and the teratozoospermia index (TZI) were calculated according to the "WHO laboratory manual…" [3]. The sperm defects with a frequency of less than 1% (double tail and thin midpiece) are not presented in the Tables and the manuscript.

2.4 Age Effects on Sperm Morphology

To investigate the effects of aging on sperm morphologies, all subjects were divided into the following age groups: 18–19 years (n = 222, 17.6%), 20–24 years (n = 530, 41.9%), 25–29 years (n = 245, 19.4%), 30–34 years (n = 124, 9.8%), 35–40 years (n = 95, 7.5%), and >40 years (n = 48, 3.8%).

2.5 Statistical Analysis

A statistical analysis of the obtained data was performed using the statistical packages "Statistica", version 8.0 (StatSoft Inc., Hamburg, Germany.). The Kolmogorov–Smirnov test was used to determine the normality of the distribution. The sperm quality parameters and the percentages of sperm morphological defects were not distributed normally. The data were best transformed by root transformation for sperm count and sperm concentration, a log transformation for the TZI, and an arcsine transformation for the percentages of progressively motile spermatozoa, morphologically normal spermatozoa, and the percentages of all sperm morphological defects. Descriptive statistics in the tables and the text are presented using untransformed data. The results are presented as the mean and standard deviation or median and 5th–95th percentiles.

To analyze the effects of aging on sperm quality parameters and the frequencies of sperm morphological defects, a one-way analysis of covariance (ANCOVA) was used. A categorical predictor (factor) in ANCOVA was age group. The period of sexual abstinence was used as a covariate. Duncan's test was used to determine the statistical significance of differences between the groups. Spearman's correlation coefficients were used to determine the correlations between age and all studied parameters.

To verify the hypothesis on the non-randomness of combinations of morphological defects in spermatozoa and to reveal non-random combinations, we estimated differences between observed frequencies of multiple sperm morphological defects (obtained from the database) and the expected frequencies of each defect. The expected frequencies for combined (multiple) sperm morphology defects were calculated as the product of the population frequencies of solitary defects included in the corresponding combinations. Statistical significance of the differences was estimated by Chi-square test.

Chi-square analysis was used to estimate differences in the frequencies of the multiple sperm morphological defects between the subjects belonging to different age groups and men with patho- and normospermia.

A p value < 0.05 was regarded as statistically significant.

3. Results

3.1 Total Frequency of Morphological Sperm Defects in the Entire Population

Predominantly, the sperm morphological defects (one or more) were found in the sperm head (46.5 $\pm$ 9.4%) or

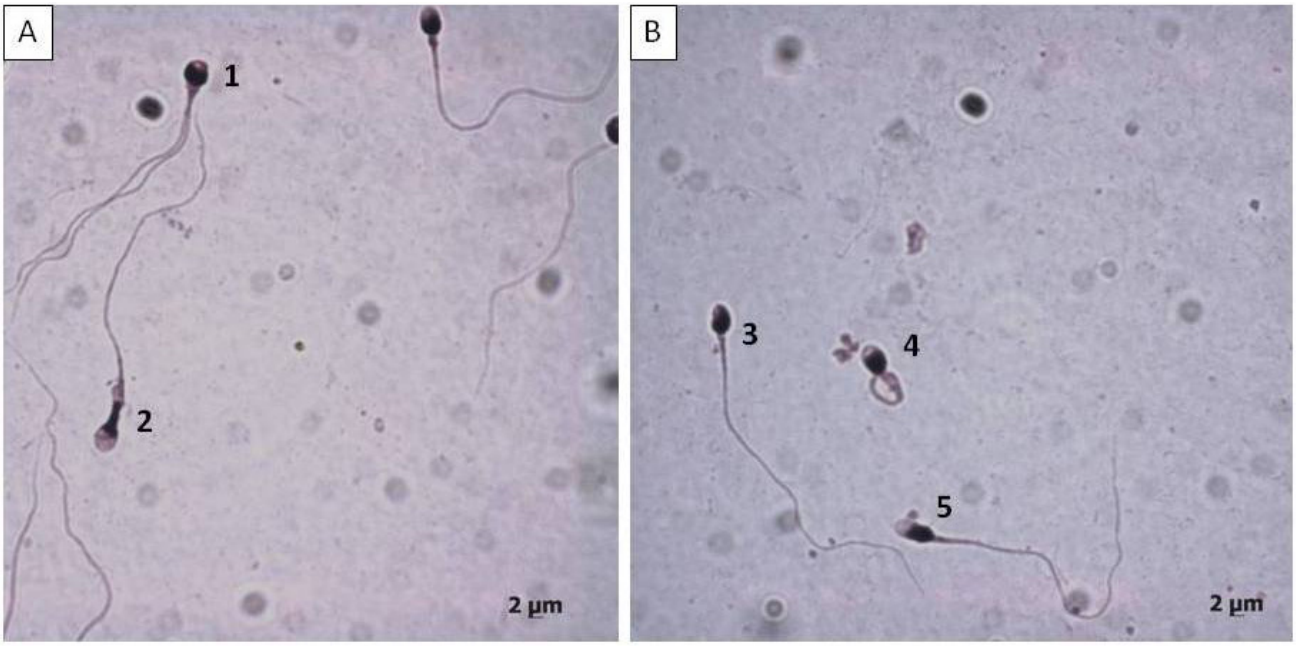

Fig. 1. Some sperm morphological defects observed in participants A and B. 1. Round head with abnormal (small) acrosome and double tail. 2. Pyriform head. 3. Amorphous head with abnormal (small) acrosome. 4. Abnormal (small) acrosome and coiled tail. 5. Elongated head. Sperm morphology was assessed with Diff-Quick staining. Light microscopy imaging of sperm under ×1000 magnification with oil immersion.

revealed simultaneously in the head and midpiece (27.3 ± 7.8%), as well as in the head and tail (10.0 ± 5.6%). More rarely, the sperm abnormalities were localized in the midpiece (4.1 ± 2.9%), and tail (1.49 ± 1.5%), or revealed simultaneously in the midpiece and tail (0.4 ± 0.6%), as well as in all three parts of the spermatozoon (3.53 ± 2.66%). The total frequencies of the sperm morphological defects in the entire population are presented in Table 1. Amorphous heads were the most frequently observed among the head defects. Among the midpiece defects, the asymmetrical neck insertion was the highest observed, while the coiled tail was the highest among the tail defects.

3.2 Age-Related Changes in the Total Frequencies of Sperm Morphology Defects

An analysis of the covariance showed the significant influence of age on the percentages of several sperm morphological defects (**Supplementary Table 1**). The percentages of spermatozoa with defects observed simultaneously in the head and tail (Fig. 2), as well as sperm in the coiled tails (Fig. 3), were higher ($p < 0.001$, Duncan test) in men older than 40 years, compared to the younger participants. The percentages of sperm with short tails in men belonging to the age groups 30–34 years, 35–40 years, and >40 years old were higher ($p < 0.05$, Duncan test) compared to the younger subjects (Fig. 4).

The frequencies of several sperm morphological defects in men older than 40 years were weakly ($p < 0.05$) reduced compared to in the younger subjects (**Supplementary Table 1**). The percentages of spermatozoa with defects observed simultaneously in the head and midpiece and sperm with thicker midpieces were lower in men aged >40 years, compared to the participants belonging to the age groups of 18–19 years and 20–24 years. The percentage of round heads in men older than 40 years was lower than in the younger subjects. The proportion of sperm with amorphous heads in men older than 40 years was lower compared to men aged 25–29 years.

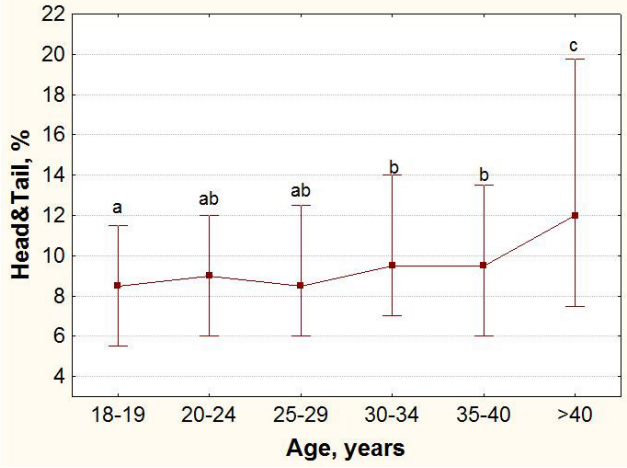

Fig. 2. The effect of age on the percentages of sperm with defects observed simultaneously in head and tail. Points indicate median and the bars depict the interquartile range; a, b, c: comparisons with different superscripts are significant ($p < 0.05$).

Table 1. Total incidences of sperm morphological defects in the entire population.

Parameters	Mean (SD)	Median (5th–95th percentile)
Morphologically normal sperm, %	6.7 (3.54)	6.5 (1.5–13.0)
Teratozoospermia index	1.48 (0.13)	1.47 (1.3–1.7)
Head abnormalities		
Amorphous head, %	62.73 (15.17)	63 (36.5–87.0)
Pyriform head, %	9.13 (9.59)	6.00 (0.5–29.00)
Elongated head, %	11.49 (9.51)	9.00 (1.0–30.5)
Round head, %	2.3 (2.86)	1.50 (0.0–8.0)
Vacuolated head, %	10.81 (7.02)	9.50 (2.0–24.0)
Acrosome defect, %	18.84 (12.06)	16.00 (5.5–43.6)
Midpiece abnormalities and ERC		
Bent neck, %	5.92 (4.07)	5.00 (1.5–13.5)
Asymmetrical neck insertion, %	17.95 (7.18)	17.50 (7.5–30.5)
Thick midpiece, %	6.23 (3.53)	5.50 (1.5–12.5)
ERC, %	7.59 (4.60)	6.50 (2–15.5)
Tail abnormalities		
Coiled tail, %	10.76 (6.53)	9.00 (3.0–23.5)
Short tail, %	3.44 (3.07)	2.50 (0.5–9.5)

Note. SD, standard deviation; ERC, excess residual cytoplasm. Sperm defects with a frequency of less than 1% (double tail and thin midpiece) are not presented in Table 1.

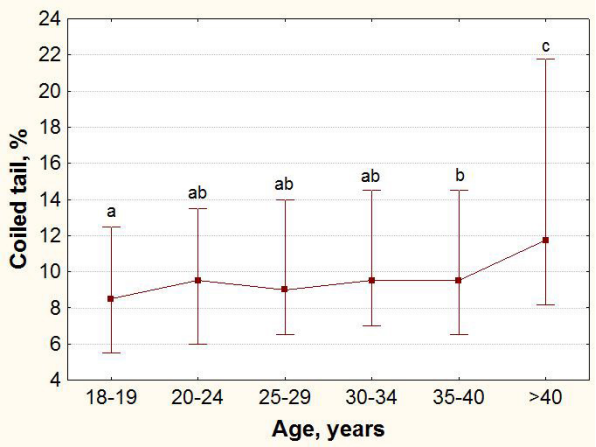

Fig. 3. The effect of age on the percentages of sperm with coiled tails. Points indicate median and the bars depict the interquartile range; a, b, c: comparisons with different superscripts are significant ($p < 0.05$).

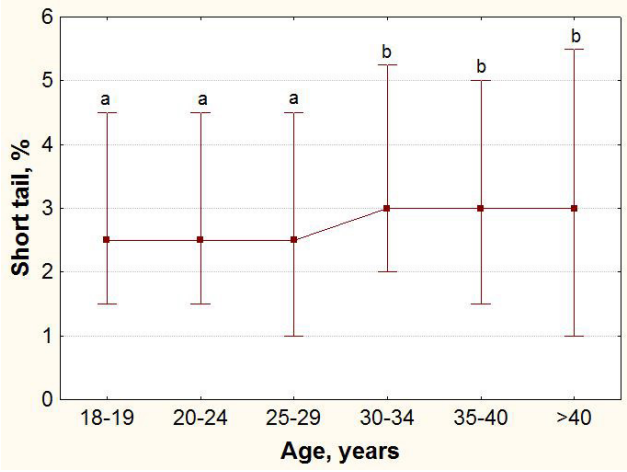

Fig. 4. The effect of age on the percentages of sperm with short tails. Points indicate median and the bars depict the interquartile range; a, b: comparisons with different superscripts are significant ($p < 0.05$).

There were no significant age effects on the percentage of morphologically normal sperm and the TZI, as well as the incidences of pyriform, elongated, vacuolated heads, acrosome defects, bent heads, ERC, asymmetrical neck insertions, and thin midpiece (**Supplementary Table 1**).

Spearman's correlation analysis revealed only linear trends of age-related variability in sperm morphologies, thereby indicating weak but statistically significant ($p < 0.05$) positive correlations between age and the percentages of elongated heads (r = 0.07), coiled tails (r = 0.10), and sperm with defects observed simultaneously in the head and tail (r = 0.11). Weak, significantly ($p < 0.05$) negative correlations were observed between age and the incidences of round heads (r = –0.06), thick midpieces (r = –0.10), double tails (r= –0.07), and sperm with defects observed simultaneously in the head and midpiece (r = –0.08). The results of the correlation analysis are presented in **Supplementary Table 2**.

3.3 Analysis of Multiple Sperm Morphological Defects in the Studied Population

Between one and seven types of morphological defects were observed in one abnormal spermatozoon (on average, 3.21 different defects). Overall, 804 variants of sperm morphological defects were revealed, including 14 single defects, and 790 different combinations of several (two or more) morphological anomalies.

Based on our experimental data, the nature of multiple morphological defects (when two or more sperm defects are observed in the same spermatozoon) can be analyzed. In particular, we verified the hypothesis that the revealed frequencies of the multiple morphological anomalies are not random frequency combinations of the solitary independent morphological abnormalities. In this case, we believe that the interrelated disturbances in the spermiogenesis processes underlie the appearance of multiple sperm morphological defects. The expected frequencies of the multiple morphological defects were calculated as the product of the frequencies of the solitary defects included in the corresponding combination. Chi-square analysis showed that the observed numbers of sperm with multiple morphological anomalies strongly ($p < 0.0001$, chi-square test) exceeded the expected sperm numbers in 48 cases (**Supplementary Table 3**). Therefore, combinations of two or more sperm morphological defects are not random combinations of independent solitary defects in these cases. It was shown that amorphous, elongated, and pyriform heads were non-randomly combined with the vacuolated head, abnormal acrosome, bent head, asymmetrical neck insertion, ERC, thick midpieces, coiled, and short tails. Round heads were non-randomly combined with asymmetrical neck insertions, thick midpieces, and coiled tails.

The following combinations of sperm morphological defects differed more strongly from the expected numbers (according to the chi-square values, top 5): amorphous head, abnormal acrosome, and asymmetrical neck insertion ($\chi^2{}_1 = 24.97 \times 10^5, p < 0.0001$), amorphous head and abnormal acrosome ($\chi^2{}_1 = 7.65 \times 10^5, p < 0.0001$), elongated head and abnormal acrosome ($\chi^2{}_1 = 4.08 \times 10^5, p < 0.0001$), pyriform head and ERC ($\chi^2{}_1 = 3.13 \times 10^5, p < 0.0001$), and amorphous head, vacuolated head, and coiled tail ($\chi^2{}_1 = 2.60 \times 10^5, p < 0.0001$).

3.4 Influence of Age on the Frequencies of Multiple Sperm Morphological Defects

We performed an analysis of the frequencies of non-random multiple sperm morphology defects in men belonging to contrasting age groups: 18–29 age (without any age differences in total frequencies of sperm morphology defects) and older than 40 years when the increased frequency of the tail defects was observed. The data are presented in **Supplementary Table 4**. It was shown that the frequencies of the coiled tail combined with amorphous, elongated, and vacuolated heads, as well as elongated heads combined with ERC and thick midpieces were higher in the subjects older than 40 years, compared to the participants belonging to the 18–29 years age groups. However, the frequencies of amorphous and pyriform heads combined with asymmetrical neck insertions and thick midpieces were higher in men aged 18–29 years compared with men older than 40 years.

3.5 The Frequencies of Multiple Sperm Morphological Defects in Men with Pathozoospermia and Normospermia

Analysis of the incidences of non-random combinations of sperm morphological defects in the subjects with pathozoospermia and normospermia was performed. The data are presented in **Supplementary Table 5**. It was shown that the frequencies of pyriform, elongated heads combined with other defects (vacuolated head, abnormal acrosome, bent head, asymmetrical neck insertion, ERC, thick midpieces, coiled, and short tails) as well as amorphous heads in combination with several other defects (abnormal acrosome, bent head, coiled tail, and short tail) were higher in men with pathozoospermia compared to the subjects with normospermia. In contrast, the frequencies of amorphous heads in combination with the presence of ERC, asymmetrical neck insertion, and thick midpieces were higher in the normospermia group compared with the pathozoospermia group.

4. Discussion

The paper presents the results of the first large-scale study of age-related changes in the frequencies of sperm morphological defects in Russian men. The detailed investigation of sperm morphology by determining the proportions of different sperm morphological defects showed that the frequencies of coiled and short tails were increased in men older than 40 years. However, aging did not affect the percentage of morphologically normal sperm and the teratozoospermia index.

The evidence on the relationships between sperm morphology and age remains contradictory. Some studies have reported that the proportion of sperm with normal morphology decreased in older men [10,53], but others did not reveal any age-related alterations in the percentage of morphologically normal sperm [41,54], similar to our study. The facts obtained in our study supplement the available evidence on the impairment of spermatogenesis in men over 40 years old and suggest that the processes of spermiogenesis and sperm maturation related to sperm tail formation can deteriorate with age, resulting in increased incidences of spermatozoa with short and coiled tails. An increased proportion of tail defects was associated with delayed embryo development in the early stages and a decrease in the frequency of implantation rates in IVF programs [24]. An elevated proportion of sperm with a coiled tail was related to an increase in the likelihood of delayed conception [15]. Therefore, the elevated frequencies of sperm with short and coiled tails could contribute to the observed decline in fertility in men after 40 years [38] and led to an increased risk of abnormal embryo development. These data indicate the need to study the molecular mechanisms of tail defect formation in older men.

It should be noted that aging and the cumulative effect of negative external factors (smoking, environmental pollution, obesity, etc.) during life affect the testis, epididymis,

and the accessory reproductive glands, thereby influencing the testicular and post-testicular development of spermatozoa. The precise relationships between cytoskeleton structures of spermatids and surrounding Sertoli cells as well as protein traffic in spermatids are known to be crucial for sperm head shaping and tail formation [5]. Deteriorations in these fine morphogenetic processes due to oxidative stress caused by aging [41], as well as exposition to environmental toxicants or negative lifestyle factors could result in the appearance of sperm morphological anomalies.

During post-testicular maturation, spermatozoa undergo several morphological and ultrastructural changes, including the final elimination of residual cytoplasm and chromatin compaction [55,56]. Moreover, macrophages in the epididymis can selectively eliminate defective spermatozoa, thereby decreasing the percentage of abnormal sperm in the ejaculate [57]. Impaired spermatozoa maturation in the epididymis results in increased incidences of coiled tails as a consequence of elevated membrane permeability and hypoosmotic stress in the spermatozoa, as well as oxidation of the outer dense fibers and fibrous sheath, which are rich in sulfhydryl groups that are turned into restraining S–S bonds [51]. It was shown that the epididymal structure and function were affected by environmental pollutants [58] and smoking [59]. Obesity and metabolic syndrome could impair the function of the epididymis, seminal vesicles, and prostate due to the accumulation of inflammatory cytokines in the reproductive tract [60]. Aging was related to changes in the epididymal morphological structure [61] and gene expression [62], as shown in the Brown Norway rat as a model of the male reproductive senescence. Given these data, it can be assumed that in men older than 40 years, the function of the epididymis is disturbed due to aging and the cumulative effect of negative environmental and lifestyle factors during life (obesity, smoking, environmental pollutants, etc.), which results in an increased incidence of tail abnormalities. Evidence in the literature and from our study indicates the importance of careful sperm morphology estimations by counting coiled sperm tails during an ejaculate assessment in men older than 40 years. This anomaly is easily recognized when examining an ejaculate smear under a light microscope.

When analyzing the frequencies of spermatozoa with different combinations of morphological abnormalities, it was shown that several combinations of two or more morphological defects in the same spermatozoon are not random combinations of independent single defects. The presence of head defects (amorphous, pyriform, and elongated heads) increased the probability of the appearance of vacuoles in postacrosomal zone (or abnormal size and/or number of vacuoles), acrosome defect, ERC, any defects of midpiece, and tail in the same spermatozoon. This phenomenon is assumed to be caused by the fact that head elongation is the important spermiogenesis stage, when, in addition to head shaping, chromatin reorganization [9,63], and tail assembly [5] are performed. Therefore, any deterioration at this stage results in not only misshapen heads but also the appearance of vacuoles as indicators of impaired chromatin packaging [49], as well as midpiece and tail defects. Moreover, the frequencies of sperm morphological defects are probably related to the overall efficiency of the functioning germinal epithelium. Therefore, any deterioration of it could result in an impairment of the spermiogenesis process related to the morphogenesis of the head, midpiece, and tail.

It is interesting that the degree of differences varied greatly between the observed frequencies of spermatozoa with certain combinations of defects and the expected values (according to the chi-square criterion). This fact is probably caused by the varying degree of severity of the relationship between the appearances of certain anomalies. Several defects, such as pyriform head and ERC, elongated head and abnormal acrosome, and amorphous head and acrosome defect, were more closely related compared to others. This morphological pattern suggests that these defects are the results of deterioration of the general processes of spermiogenesis related to the sperm head shaping, acrosome morphogenesis, and removal of excess cytoplasm. In particular, the simultaneous presence of a pyriform head (head with narrowed and elongated postacrosomal zone) and ERC in the same spermatozoa may be caused by the impaired functioning of the manchett-transient cytoskeleton structure in spermatids. This structure is known to be necessary for the formation of the postacrosomal region, the removal of excess cytoplasm, and the formation of the tail [5,64,65].

The present study showed that the frequency of several multiple morphological defects in spermatozoa was related to the sperm quality (pathozoospermia and normospermia). These data indicate that certain coordinated morphogenetic processes during spermiogenesis can be influenced by testicular dysfunction. In particular, the protein traffic in spermatids is important for chromatin compaction and tail formation [66]. In addition to the disturbed epididymal maturation discussed above, any impairments in this traffic could result in an increased occurrence of the vacuolated head combined with the coiled tail in men older than 40 years. The total proportions of many sperm morphological defects were higher in men with pathozoospermia compared to the subjects with normospermia. These findings correspond to the previously obtained results [36]. The increased incidence of morphological defects and their combinations is probably caused by impaired spermatogenesis in the subjects with pathozoospermia.

It should be noted that amorphous heads combined with several other defects (asymmetrical neck insertion and thick midpiece) were more common in normospermic men in comparison with pathozoospermic subjects. These results could be explained by the fact that in the sperm morphology classification provided by the “WHO manual…” [3], some sperm morphological forms (spermatozoa with minor violations in shape and size) should be considered abnormal, while slightly misshapen heads should be classi-

fied as amorphous. However, these borderline head shapes as well as several midpiece and tail defects can also appear in humans with the normal germ epithelium function. However, the deeply misshapen heads (pyriform, elongated, and round) are more common in men with pathozoospermia and are better markers of the impaired spermatogenesis efficiency. These results suggest the need to include borderline defects in a separate category when assessing sperm morphology.

The close relationship between the appearance of different morphological abnormalities in spermatozoa, its relationship with age, and sperm quality suggest the need for further study of the molecular processes underlying the formation of sperm morphological defects. The studies in knockout animals [5] and monomorphic teratozoospermia in humans [67–69] revealed that mutations in some genes that control spermiogenesis can lead to the appearance of specific morphological defects. However, the relationships between genetic factors, environment, and sperm morphology remain poorly understood [70] and should be rigorously investigated.

5. Conclusions

This paper presents the results of the first large-scale study of age-related changes in the frequencies of various morphological sperm defects. It was shown that the proportion of tail defects (shorts and coiled tails) increased in men older than 40 years. These anomalies in sperm morphogenesis are probably caused by age-related disturbances to sperm maturation in the epididymis, resulting from aging and cumulative effects of negative environmental and lifestyle factors during a man's life.

It was revealed that several combinations of two or more sperm morphological defects in the same spermatozoon are not random combinations of independent single defects. The existence of head defects (amorphous, pyriform, and elongated heads) increased the probability of the appearance of vacuoles in the postacrosomal zone (or abnormal size and/or number of vacuoles), acrosome defects, excess residual cytoplasm (ERC), and any midpiece and tail defects in the same spermatozoon. Here, the frequencies of several combinations of sperm morphological defects were related to the increased men's age (combinations of the coiled tails with amorphous, elongated, and vacuolated heads) and sperm quality (normo- and pathozoospermia). The sperm morphological defects with strongly misshapen heads (pyriform, elongated, and round) were more common in men with pathozoospermia compared to normospermic subjects. The obtained evidence suggests the existence of certain coordinated morphogenetic processes during spermiogenesis, which could be influenced by age-related factors and impaired testicular function, thereby causing the appearance of multiple sperm morphological defects.

Abbreviations

ERC, excess residual cytoplasm; TZI, teratozoospermia index.

Availability of Data and Materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Author Contributions

LO, and AO designed the research study. MK, LO, and AO performed the research. MK and AO analyzed the data. MK and AO wrote the manuscript. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

The Ethics Committee of the Federal Research Center 'Institute of Cytology and Genetics', the Siberian Branch of the Russian Academy of Sciences approved the study (protocol № 160 and date of approval 17 September 2020).

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Conflict of Interest

The authors declare no conflict of interest.

Supplementary Material

Supplementary material associated with this article can be found, in the online version, at https://doi.org/10.31083/j.fbs1503012.

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