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SPERM DNA FRAGMENTATION: ETIOLOGY, PATHOGENESIS, THE INFLUENCE ON REPRODUCTIVE FUNCTION

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The literature review of sperm DNA fragmentation is evaluated. Russian and foreign literary data over the past 10 years, including fundamental researching of the evaluation of the gametes genome integrity, are analyzed. The main etiological factors and the possible reasons of the DNA breaks formation on the different stages of spermatogenesis are described. The influence of the sperm oxidative stress reaction to the DNA integrity is analyzed. The relationship between DNA fragmentation of spermatozoa pregnancy and live birth rates in the assisted reproductive techniqueare noted. Risk of the recurrent pregnancy losses in male infertility cases with the sperm DNA damage is presented. Review confirms the significant prognostic value of sperm DNA fragmentation detection in infertility cases. Further studies in evaluation of pathogenesis of sperm DNA have a clinical interest to reproductive health physicians.

Keywords: male infertility; sperm DNA fragmentation; literaturereview; chromatin; varicocele; oxidative stress; reactive oxygen species; assisted reproductive technique.

ДНК-ФРАГМЕНТАЦИЯ СПЕРМАТОЗОИДОВ: ЭТИОЛОГИЯ, ПАТОГЕНЕЗ, ВЛИЯНИЕ НА РЕПРОДУКТИВНУЮ ФУНКЦИЮ

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Обзор литературы посвящен ДНК-фрагментации сперматозоидов. Проанализированы отечественные и зарубежные литературные источники за последние 10 лет, а также фундаментальные данные по оценке целостности генома гамет. Описаны возможные механизмы формирования разрывов ДНК на различных этапах сперматогенеза, основные этиологические факторы. Отдельным пунктом выделена значимость избыточного оксидативного стресса эякулята в генезе патоспермии и повреждения хроматина. В обзоре представлены современные данные о взаимосвязи между ДНК-фрагментацией сперматозоидов и частотой наступления беременности и родов при лечении методами вспомогательных репродуктивных технологий. Описаны риски потерь беременности при высоком показателе фрагментации ДНК. В статье подчеркнута диагностическая значимость определения нарушений структуры хроматина при мужском факторе бесплодия. Дальнейшие исследования в области патогенеза разрывов ДНК гамет представляют клинический интерес для специалистов, занимающихся репродуктивным здоровьем.

Ключевые слова: мужское бесплодие; ДНК-фрагментация сперматозоидов; обзор литературы; хроматин; варикоцеле; оксидативный стресс; активные формы кислорода; вспомогательные репродуктивные технологии.

INTRODUCTION

The decline in the birth rate in developed countries is one of the most significant contemporary medical and socio-economic problems. The frequency of infertile marriages is about 10% among married couples in Europe, 11%–15% in the USA, about 17% in Canada, and 15.4% in Australia. In Russia, the infertility rate in marriage exceeds 17% [1]. According to the Federal State Statistics Service, a decrease in the birth rate has been registered

in the Russian Federation since 2015. The birth rate in 2018 was 10.7 births per 1000 population, ranking 184th in the world [2]. According to epidemiological studies, the male population's reproductive potential is decreasing, which tends to increase the number of childless couples increase [1].

Currently, a spermogram is used to assess the fertility status of men. However, the quantitative and qualitative parameters of the ejaculate do not guarantee the birth of a healthy child in 100% of cases. The sperm DNA fragmentation (SDF) test enables the integrity of the genome to be determined, which is an essential element that affects not only the frequency of pregnancy but also its course. Thus, the SDF is becoming a more accurate biological marker of male fertility [3–5].

The widespread use of assisted reproductive technologies (ART) to overcome infertility leads to the leveling of natural selection. It increases the risks of fertilization of an oocyte by a sperm with a damaged chromatin structure. The consequence may be spontaneous abortions or the birth of children with genetic, developmental defects [5–7]. The above information indicates the relevance of the subject of this review.

HISTORICAL BACKGROUND

The study of the sperm chromatin structure was started in the middle of the twentieth century. In 1946, A. Pollister and A. Mirsky described protamine complexes surrounding trout spermatozoa's DNA [8]. In 1953, along with discovering the double-helical structure of DNA (J. Watson and F. Crick), Leuchtenberger et al. [9] noted that gametes' chromatin structure in infertile patients has greater deviations than fertile men.

In 1970, in experimental work, Ringertz et al. assessed the thermal effect on bovine spermatozoa and determined DNA denaturation using acridine orange staining and microfluorimetry. The study results showed that spermatozoa had increased resistance in the phase of spermatogenesis [10]. In 1976, in an experiment, Alfert revealed that histones are replaced by protamines during spermatozoa maturation in the postmeiotic phase [11]. These fundamental studies have become the starting point for the continuous process of studying the male gamete's genome.

Based on the different sensitivity of damaged and intact DNA to acid denaturation, an acridine orange test (AO) was developed. After drying with atmospheric air, smears of ejaculate are fixed with a methanol-acetic acid solution and stained with AO (fluorochrome). The number of chromatin breaks correlates directly with denaturation, reflected in the transition of DNA from the double-stranded to the single-stranded form. The advantages of the test are its low cost and minimal technical difficulties in execution. However, the test duration can lead to artifacts in obtaining results, limiting AO's utility in clinical practice [12, 13].

In the 1980s, Evenson et al. developed a sperm chromatin structure assay (SCSA), a method for identifying SDF using flow cytometry. The sperm DNA is denatured with acid at the breaks in the strands and then stained with a fluorescent cationic stain, AO. The test detects single-and double-stranded DNA breaks and the percentage of sperm with an immature nucleus and an abnormal chromatin structure. These characteristics determine the assay's high diagnostic sensitivity, which enables its use in clinical practice [14].

In the 1990s, the Comet Assay method, based on cell mini-electrophoresis, was introduced. It is used to determine the content of high- and low-molecular-weight DNA in the latter area, which resembles the tail of a comet. Literature data indicate the high clinical and diagnostic value of the Comet Assay [15].

At the same time, the TUNEL method (terminal deoxynucleotidyl transferase dUTP nick end labeling) was developed. This method is based on direct labeling of DNA breaks with a fluorochrome and measures the luminescence intensity. Terminal deoxynucleotidyl transferase indicates a DNA strand break with fluorescent dUTP nucleotides [16]. Numerous studies have demonstrated the high sensitivity of TUNEL in predicting ART procedures' success [4–7, 16].

An indirect method for determining SDF is the sperm chromatin dispersion (SCD) test. It uses specific fluorochromes and a fluorescence microscope to enable the dispersed DNA to be viewed as a halo surrounding the nuclei. When chromatin is fragmented, mild dispersion is noted, which results in mild luminescence. SCD is characterized by dividing cells into categories according to the degree of SDF [17]. The Halosperm test is a modification of the SCD test. It is based on the study of spermatozoa chromatin density, which evaluates the results and

possibly identifies an intact tail using microscopy in a bright field. The improved technique facilitates the differentiation of gametes from other types of ejaculate cells [18].

In 2006, Li et al. developed the γH2AX test for detecting double-stranded DNA breaks in gametes [19]. Several kinase proteins induce phosphorylation of Ser139 on histone H2AX. Phosphospecific antibodies can recognize a phosphorylated serine residue as quantified by a flow cytometer. Despite the pronounced DNA protaminization in the postmeiotic maturation phase of spermatozoa, a small fraction containing H2AX histones remains in the nucleosome (about 15%). This method enables determining double-stranded DNA breaks in gametes. In a study conducted in 2015 by Garolla et al., the method's clinical significance was assessed by analyzing the efficiency of Intra Cytoplasmic Sperm Injection (ICSI) procedures. The results of the work demonstrated a significant correlation between the SDF values and the frequency of pregnancy. The disadvantages of the test include its laboriousness and the time required to perform it [20].

DW1 is a method for detecting single- and double-stranded DNA breaks in spermatozoa. It is based on the use of a synthetic peptide consisting of 21 amino acids and linked by a fluorescent stain rhodamine B. The latter interacts with the critical region of p53. The results of a comparative assessment of the DW1 and TUNEL showed a significant correlation. Although the study requires removing the sperm membrane using a detergent, according to the authors, further improvement of this technique will enable selecting viable gametes with intact DNA to perform the ICSI procedure [21, 22].

MODELING OF CHROMATIN AT DIFFERENT STAGES OF SPERMATOGENESIS. PATHOPHYSIOLOGICAL ASPECTS OF DNA FRAGMENTATION OF SPERMATOZOA

Spermatozoa are highly differentiated cells with a unique ultrastructure. Their compact DNA arrangement protects against various damaging factors. The types of structural changes in sperm chromatin are represented by SDF, nuclear protein defects, and rearrangements in the sequence of DNA regions. They can be generated during spermatogenesis, spermiogenesis, transit along the vas deferens or after ejaculation [5, 23–26].

During spermatogenesis, gametes are formed from spermatogonia or primitive sperm cells. The process is divided into three main stages, namely, premeiotic, meiotic, and postmeiotic. Specific chromatin changes occurring at different stages of gametogenesis can be considered a source of DNA breaks [23–27].

The premeiotic phase is represented by mitotic proliferation. The primitive sperm cells are located on the basement membrane of the seminiferous tubules. They are of two morphological types, A and B. Type A primitive sperm cells are divided according to chromatin condensation in the nucleus into dark (condensed chromatin) and light (diffuse chromatin). Dark type A primitive sperm cells constitute a reserve pool of cells without proliferative activity. They undergo mitotic changes with a sharp decrease in the density of primitive sperm cells in the testes. Light type A primitive sperm cells continuously divide by mitosis and form an equal number of daughter cells of type A and type B. As a result of reduction division, either two type B primitive sperm cells are formed, or one type B primitive sperm cell and one type A light primitive sperm cell are formed. Type B primitive sperm cells have a light, rounded nucleus with well-condensed chromatin. As a result of several mitotic divisions, the latter differentiate into first-order spermatocytes [23–27].

In pachytene of meiosis, chromosomes are subject to a process of homologous recombination, and somatic histones are replaced by testis-specific ones. At these stages, unformed gametes are especially sensitive to external toxicants, and unrepaired DNA changes can be fixed in the form of mutations after replication [4, 5, 24–27].

During the postmeiotic phase, chromatin remodeling occurs, associated with the loss of histones and their replacement by specific transient proteins. The latter proteins are subsequently replaced by protamines, which are arginine-rich proteins linked by disulfide bonds. This leads to the compaction of chromatin and its transformation into a unique structure of the nucleus of the sperm. It is in the postmeiotic stage that remodeling repair of DNA breaks occurs. However, some of them remain unrepaired, and as a result, SDF occurs [4, 5, 24–27].

Sperm DNA is represented by protamines by 85%–90%. The remaining 10%–15% of the sites are represented by histones. This creates the availability of chromatin for transcription after oocyte fertilization [23, 25, 27]. Sperm chromatin remodeling results in the production of temporary nicks in the DNA. The triggering mechanism of this process is histone modification, such as specific methylation, acetylation, phosphorylation, ubiquitination, and hyperacetylation [25, 27].

The activation of endogenous nucleases (topoisomerase types I and II) catalyzes the DNA strand's unwinding, breaking the supercoil, followed by repair. Topoisomerase I induces multiple single-stranded and double-stranded breaks in sperm chromosomes. This process helps to solve the problem of DNA supercoiling and facilitates the removal of histones during protaminization. Topoisomerase II is the main enzyme in DNA repair in elongated spermatids. It is inhibited by the enzyme poly(ADP-ribose)polymerase, activated in turn by DNA breaks. Apoptosis serves to eliminate cells with unrepaired DNA [24–27].

The onset of chromatin condensation is accompanied by an increase in the number of DNA breaks, which are then repaired. Their ligation is provided by the inserted transition nuclear protein (TNP) TP1-TP4. The latter, in turn, bind to DNA, facilitating interaction with protamines. Their function consists of facilitating chromatin remodeling and condensation, and repair of DNA breaks [4, 26, 27]. At the final stage of compaction during spermatozoa's epididymal transport, the thiol groups of protamines are oxidized, forming numerous internal and external disulfide bonds between cysteine residues. They thicken chromatin and stabilize the sperm nucleus. This is significant in head formation, inactivation of genome transcription, and protection and stabilization of gamete DNA. Until the completion of spermatogenesis, temporary nicks are restored. Otherwise, an extra fragment of DNA may appear in the sperm. Unrepaired breaks occurring during chromatin remodeling are considered one of the primary sources of SDF, and their presence indicates incomplete spermatogenesis [26, 27].

Thus, the genome of male gametes has a unique structure and is organized in a specific way that distinguishes it from the chromatin of all types of somatic cells. The volume of the sperm nucleus is six times less than that of the somatic cell nucleus. Supercoiling provides stability, transcriptional inertness, and protection of the genome during passage along the vas deferens [5, 23, 25–27].

OXIDATIVE STRESS AND SPERMATIC ANTIOXIDANT SYSTEM. APOPTOSIS

According to numerous studies, excessive oxidative stress (OS) plays a key pathogenetic role in sperm DNA damage [4, 5, 23, 26, 28–30].

Reactive oxygen species (ROS) are highly reactive oxidants classified as free radicals (FR). They are continuously generated due to cell metabolism and, in physiological quantities, play an important role in chromatin condensation, hyperactivation, and spermatozoa capacitation [26–29]. Pathological OS is a consequence of an imbalance between ROS production and spermatic antioxidant activity. The main sources of FRs in sperm are immature germ cells, neutrophilic leukocytes, and morphologically abnormal spermatozoa. The residual sperm cytoplasm retention can also lead to ROS hyperproduction, associated with the SDF risks.

Unlike somatic cells, germinal cells are more vulnerable to OS due to the lack of the necessary cytoplasmic enzyme repair system. In addition, the cytoplasmic membrane contains a large amount of polyunsaturated fatty acids and membrane-bound nicotinamide-adenine dinucleotide phosphate (NADP) oxidase-5, which makes gametes susceptible to FR attacks. NADP oxidase has been identified as a ROS production source in ejaculated spermatozoa [4, 5, 23, 25, 26, 28, 30]. The increased FR content leads to membrane destruction, which ultimately decreases spermatozoa's mobility and fertilizing ability. Damage to the genome by excess OS can manifest itself in the form of chromatin denaturation, base modification, point mutations, gene polymorphism, deletions, reading frameshift, and single- and double-stranded DNA strand breaks [5, 28–33].

One mechanism of sperm DNA damage is abortive (incomplete) apoptosis during spermatogenesis. Through apoptosis, germ cells with various disorders and damage are eliminated, which is a physiological process [5, 25, 26, 29, 32]. It is assumed that apoptosis during spermatogenesis can perform two functions, namely, the numerical limitation of the population of spermatogonial cells and, apparently, the selective death of abnormal spermatozoa. Moreover, the mechanism of apoptosis during sper-

miogenesis may be less associated with cell death. However, play a significant role in the cytoplasm separation process during the last stages of sperm maturation. Accordingly, suppression of this process due to various causes can lead to SDF risks [4, 5, 26, 27, 29–32].

The presence of apoptotic bodies of testicular origin in the sperm of an infertile patient indicates that apoptosis is triggered mainly in the testis [25, 34]. However, the detection of a larger amount of fragmented DNA in ejaculatory spermatozoa, compared with testicular spermatozoa, indicates the possible presence of apoptotic stimuli during the gametes' transit along the vas deferens [25, 34].

Normally, the sperm genome is protected from excess OS by the seminal plasma's antioxidant system. It includes non-enzymatic (vitamins A, C, E, ascorbate, glutathione pyruvate, glycine, zinc, others) and enzymatic antioxidants (superoxide dismutase [SOD], glutathione peroxidase [GPX], and catalase [CAT]) [28, 30, 31].

The combination of the antioxidant profile determined by the activities of SOD, GPX, and CAT and the lipid peroxidation biomarker (malonyldialdehyde) is the best indicator of OS. A reduced antioxidant profile and an increased concentration of malonyldialdehyde correlate with SDF [28, 31].

ETIOLOGICAL FACTORS OF DNA FRAGMENTATION OF SPERMATOZOA

A high level of SDF is often a form of male infertility which is difficult to overcome. The main etiological factors include older paternal age [35–37], varicocele [37, 38], increased levels of radiation and ionizing radiation, sexual abstinence, the influence of Wi-Fi waves [39], infectious and inflammatory diseases of the reproductive tract [5, 23, 28, 30, 37], chemotherapy and radiotherapy, oncological processes, contact with heavy metals, metabolic disorders, diabetes mellitus, the intake of gametotoxic drugs [40], spinal cord injuries, and hyperthermia [23]. The negative influence of tobacco smoking, narcotic drugs, and excessive alcohol consumption on the integrity of chromatin has been established [5, 23, 37]. According to Boeri et al. [41], the presence of human papillomavirus in ejaculate can negatively affect sperm motility and be a risk factor for SDF.

Analysis of recently published data revealed that up to 20% of idiopathic infertility cases were associated with SDF. It was noted that the detection frequency of fragmentation in normozoospermia, including in sperm donors, falls within a relatively wide range, from 0% to 25% [4, 5, 23, 26]. The fluctuations noted can be explained by the different nationalities and ethnicity of the donors, the requirements imposed on their state of health, the timing of sexual abstinence, and the differences in the methods of diagnosing SDF.

Damage to the sperm genome can occur during the manipulation of the ejaculate in ART laboratories (cryopreservation, storage conditions, violations of the sperm collection, and filling and pretreatment procedures). When washing the ejaculate, the seminal plasma, which has antioxidant properties, is removed. Centrifugation and long-term incubation of spermatozoa increase the probability of a harmful effect by ROS on DNA. This is especially important since spermatozoa used in in-vitro fertilization (IVF)/ICSI programs, selected only based on morphological and functional characteristics, may have a damaged DNA strand [42].

INFLUENCE OF DNA FRAGMENTATION OF SPERMATOZOA ON REPRODUCTIVE FUNCTION

Damage to the spermatozoa chromatin structure represents one of the possible causes for the inability to become pregnant naturally, can lead to disorders of embryonic development, the arrest of development, and elimination of the embryo at the early stages of embryo- and ontogenesis, and cause habitual pregnancy losses [4–7, 43–46].

Despite the oocyte's proven ability to repair sperm DNA damage during in–vitro fertilization or natural conception, oocytes can be susceptible to oxidative attack by the spermatozoa. This can result in a functionality disorder of the oocyte and the loss of mechanisms to restore mechanical defects in the male gamete DNA. A single-strand break in a DNA molecule can be repaired by a female gamete during embryogenesis. Double-stranded damage is almost impossible to correct, which inevitably leads to impaired development of the embryo or fetus [4–6, 43–46].

Sugihara et al. [47] conducted a systematic literature review to determine the significance of SDF in predicting the clinical pregnancy probability after intrauterine insemination. The work included nine studies for qualitative analysis and four studies for meta-analysis, which amounted to 940 cycles.

A low correlation between SDF and pregnancy rates was noted. The combined sensitivity and specificity of the assay were 94% (95% CI: 0.88; 0.97) and 19% (95% CI: 0.14; 0.26), respectively. It was concluded that SDF is of limited value in predicting the results of intrauterine insemination. The authors pointed out the need for further cases to assess the threshold values and stability of the SDF index over time and before and after centrifugation in a density gradient [47].

The data in the literature indicate that in vitro during IVF and ICSI procedures at a high level of SDF, risks of embryogenesis disorders and embryo development arrest exist, which reduce the clinical pregnancy probability [4–7, 43–46]. However, spermatozoa with DNA damage retain the ability to achieve fertilization. This fact explains the effect of fertilization during IVF and ICSI with increased SDF values. However, in this case, the spontaneous abortion risk increases by 2.5–4 times, regardless of the use of the standard IVF protocol or ICSI selection [5–7, 43–46].

An increase in SDF is a risk factor for the development of congenital malformations of the fetus, chromosomal abnormalities in children, the possibility of delaying physical and mental development, and a predisposition to cancer [48].

CONCLUSION

The above information emphasizes the diagnostic value of SDF determination in various forms of infertility, including habitual pregnancy loss. However, the introduction of tests for the integrity of gamete DNA into routine clinical practice often requires expensive equipment and trained personnel. In addition, the lack of uniform threshold standard values is also a significant problem.

Optimizing diagnostic measures and creating a unified examination protocol for male infertility factors are priorities to increase the world's birth rate.

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