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Comparative Evaluation of the Parameters of Sperm Apoptosis of Young and Middle-Aged Men by Flow Cytometry

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A comparative analysis of the parameters characterizing sperm apoptosis of young (27-42 years) and middle-aged (44-51 years) men was performed by flow cytometry. Irrespective of age, activity of caspase-3 and p53-mediated controlling the transmission of apoptogenic signal transmission in gametes remained stable with the formation of germ cells with delayed ($p<0.05$) cell death according to the Annexin V-FITC^{+}PI^{-} criterion (predominantly in middle-aged men). Inhibition of the transmission of a proapoptogenic stimulus mediated by membrane cell death receptors (FAS) was also observed in this group. Comparison of indicators of sperm apoptosis showed age-related features of cell death, in particular, inhibition of membrane reception triggering FAS-dependent apoptosis, which is associated with insufficient phosphatidylserine production in middle-aged men, excessive life cycle duration, and aging of spermatozoa.

Key Words: *flow cytometry; spermatozoon; caspase; p53; receptor-mediated apoptosis*

Flow cytometry is now widely used in the diagnostics and monitoring of male reproductive system diseases. Flow cytometry is a promising technique to estimate sperm quality because of its high informative value and statistical reliability as well as the opportunity to work with data obtained on a sampling that is quantitatively and qualitatively representative.

A standard spermogram includes evaluation of sperm quantity, morphology, motility, and viability. At the same time, standard tests for assessing gamete viability (eosin staining) does not allow precise evaluation of membrane damage, which creates certain difficulties in timely diagnostics of fertility disorders and infertility in men and makes therapies less effective. The use of cell technologies in andrology allows simultaneous evaluation of several parameters in one test including membrane state, morphological properties, and functional activity of the spermatozoon, expression of membrane receptors, degree of DNA fragmentation, and apoptosis.

Apoptosis plays an important role in development and regulation of male reproductive functions [1]. Defective cells in an adult are eliminated via apoptosis; defects in apoptotic death mechanism cause abnormal sperm development; male gametes die through apoptosis in female genital tracts, which allows avoiding inflammation in internal genitals. Disorders in apoptosis induction are often the reason for poor fertilization and infertility [6]. Previous studies showed that the intensity of genetically determined cell death was associated with poorer sperm quality [6]. Sperm apoptosis was shown to depend on many factors. It was revealed that patients with chronic inflammatory diseases of the reproductive system had increased concentrations of proapoptotic cytokines in ejaculates and this could induce sperm apoptosis and reduce their motility. Overexpression of FAS and FASL was detected in spermatocytes and spermatids during a chronic

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inflammatory process in reproductive organs [5]. Normally, proliferative activity of primitive spermatozoa increases with age. Disorders in the dynamics of mitochondrial networks in peritubular myoid cells were detected in aging men with normal spermatogenesis [8]. Estimation of natural sperm death was shown to be significant for preventing and eliminating reproductive disorders in men of different age under different conditions.

Thus, studies performed during the last decade have proven a significant role of apoptosis in gamete life cycle; cytometry allows obtaining relevant and sufficient data on sperm viability.

Our aim was to examine the parameters that characterized apoptosis in ejaculated sperm of men from different age groups by flow cytometry.

MATERIALS AND METHODS

The study was performed in conformity with the ethical principles for medical research involving human subjects (WMA Declaration of Helsinki, 2013). The material for the study was ejaculates of practically healthy men of young (27-42 years; reference group, n=36) and middle age (44-51 years; test group, n=24) (p<0.001). The inclusion criteria were: age from 27 to 51 years, children born in a marriage (the reference group), a married couple being infertile for not less than 5 years (test group). Exclusion criterion was participation in other studies.

Fresh sperm samples were qualitatively assessed in accordance with WHO guidelines (2010). Ejaculate was estimated with flow cytometry to examine the following indicators: $CD95^+$ (FAS) expression, p53 content, caspase-3 activity, proportion of Annexin V-$FITC^+PI^+$ and Annexin V-$FITC^+PI^-$ spermatozoa. Membrane and intracellular factors controlling apoptosis were quantitatively estimated by staining with monoclonal antibodies according to the manufacturer protocol (Becton Dickinson). Immunoassay was perfumed on FACSCalibur (BD).

Obtained results were statistically analyzed using Statistica 6.0 software (StatSoft, Inc.). The data were presented as Me (25%; 75%) and confidence interval for median (95%CI). We applied non-parametric Mann—Whitney U test was applied to evaluated significance of differences between independent samplings. The significance level for testing the null hypotheses was set at 0.05.

RESULTS

The total number of spermatozoa in the ejaculate of young men from the reference group (126.00 (115.68-193.15)×10^6) and middle-aged men from the test group (115.00 (101.71-179.91)×10^6) (p=0.828) corresponded to WHO standards (2010). A significant increase (by 1.3 times; p=0.015) in the number of immotile sperm in middle-aged men (29.0 (26.19-34.56)%) in comparison with young subjects (22.0 (17.84-26.73)%). Analysis of the spermogram showed that the percentage of morphologically normal cells in young (62.00 (49.33-63.79)%) and middle-aged men (58.00 (47.73-60.08)%) was within the reference values (p=0.53). We should note that increased concentrations of morphologically changed spermatozoa were detected in 6.7% cases among middle-aged men and in only 4.4% cases among young ones (1.5-fold difference between the groups).

Cytometry of the ejaculated semen revealed significant decrease (by 2.2 times; p=0.011) in the expression of $CD95^+$-antigens in gametes of middle-aged men (Table 1). A percentage of Annexin V-$FITC^+$-PI^-- spermatozoa was 2-fold lower in middle-aged men (p=0.045). There were no significant differences in intracellular p53 contents, caspase-3 activity, and

TABLE 1. Parameters of Sperm Apoptosis in Men of Different Age Groups (Me (25; 75%); 95%CI)

Parameter	Yong men (27-41 years) (n=36)	Middle-aged men (44-51 years) (n=24)	Mann—Whitney U test	Z test	p
$CD95^+$-spermatozoa, %	25.69 (12.20; 30.85) 18.79-27.49	11.95 (3.08; 18.44) 5.52-18.68	118.00	2.54	0.011
p53, %	10.95 (8.08; 14.68) 10.73-16.83	11.35 (6.18; 23.14) 8.81-22.43	250.00	0.04	0.965
Caspase-3, ng/ml	5.34 (2.44; 8.71) 5.03-9.64	4.18 (1.69; 4.95) 2.66-5.62	169.00	1.47	0.141
Annexin V-$FITC^+PI^-$-spermatozoa, %	1.12 (0.80; 2.52) 1.29-2.83	0.56 (0.24; 1.42) 0.37-1.51	159.50	1.99	0.045
Annexin V-$FITC^+PI^+$-spermatozoa, %	58.46 (46.59; 74.30) 51.01-64.46	55.94 (26.38; 77.90) 36.59-65.66	218.00	0.59	0.550

percentage of Annexin V-FITC$^+$PI$^+$- spermatozoa in ejaculates from men of both groups (p=0.055-0.965).

Cell death can occur as per extrinsic (receptor-mediated) or intrinsic (mitochondrial) pathway. Extrinsic apoptosis pathway involves FAS-receptor representative of the superfamily of TNF receptors (TNFRSF6). The intrinsic pathway is triggered by proapoptotic proteins of the BCL-2 family and the process involves damage to external mitochondrial membrane and cytochrome C release. The balance within FAS/FASL system guarantees adequate life cycle of the spermatozoa and any disorders in the system can lead to destructive changes in the germinal epithelium cells, changes in spermatogenesis, and infertility [5]. For instance, in case of inflammation, gametes undergo apoptosis due to imbalance between the pro- and anti-apoptotic signals. FASL induces FAS trimerization on spermatocytes and spermatids and mediates receptor-dependent apoptosis [3]. Apart from activation of FAS-mediated apoptosis, the cell can respond to exogenous or endogenous stimuli by induction of mitochondrial apoptosis. The role played by intrinsic apoptosis pathway in gametes is well proven [4]. However, the molecular mechanisms that underlie non-receptor apoptosis induced by damaging factors have not been properly studied. Effector caspase-3 is at the end of the caspase cascade and is activated in both extrinsic and intrinsic apoptosis pathways [9]. At the same time, there is evidence that caspase-3 has non-apoptotic functions. Thus, caspase-3 has been established to have a significant role at a stage of spermatids individualization. It is assumed that transition of pre-spermatogonia to their dormancy is largely driven this enzyme. This proteolytic enzyme is also confirmed to take part in andrologic pathology development including spermatogenesis disorders, asthenozoospermia, increased DNA fragmentation in spermatozoa, testicular torsion, varicocele, and immunologic infertility [7]. There is convincing evidence that p53 protein can directly interact with both anti-apoptotic and proapoptotic proteins located in the cytoplasm and mitochondrial membranes [2]. A contribution of this protein into ageing is now actively studied. Enhanced expression of p53 in aging cells has been proven in experiments [10]. The mechanisms of immunological tolerance have the leading role in the maintenance of normal life cycle of the spermatozoon; at the same time, their negative modification creates high risks of infection and tumorigenesis. Significant activity by immunocompetent cells can induce excessive immune response associated with proliferative processes.

New developing technologies such as flow cytometry provide an opportunity to solve a wide range of tasks in andrology. Changes in semen quality that develop with age have significant influence on male reproductive potential. We established that the mechanism of p53-controlled apoptosis of spermatozoa is equally preserved in healthy young and middle-aged men (p=0.965). We also revealed no significant differences in caspase-3 activity between the two examined groups (p=0.141). All men irrespective of their age had the same percentage of Annexin V-FITC$^+$PI$^+$-spermatozoa, *i.e*. cells that die through necrosis or are at the later stage of apoptosis (p=0.550). The content of Annexin V-FITC$^+$PI$^-$-spermatozoa in the ejaculate of middle-aged men was significantly lower (by 2 times; p=0.045) than in young men. Comparative assessment of ejaculated semen revealed some peculiarities in indicators associated with apoptosis induction and realization in men aged from 44 to 51 (a significant decrease in the expression of surface marker of receptor-mediated apoptosis CD95$^+$ (FAS), p=0.011).

Thus, comparative assessment of semen quality in health men by flow cytometry showed a decrease in programmed cell death associated with disorders of reproductive functions in middle-aged men.

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