



## THE INFLUENCE OF ELECTROSTATIC FIELDS ON STRUCTURAL AND FUNCTIONAL STATE OF EPIDIDYMAL SPERMATOZOIDS

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### Abstract

The influence of 200 *kV/m* electrostatic fields on processes of epididymal maturation of spermatozooids and the structural and functional state of mature gametes was investigated after one-hour effect and fractional influence for 6 hours during 6 days.

It was shown that one-hour influence of investigated field results in statistically non-reliable increase of testosterone concentration in blood and testes, as well as the amount of viable spermatozooids in cauda epididymis, while the fractional influence led to a decrease of hormone concentration in testes and to reduction of the amount of viable spermatozooids. In both cases, the decrease of cholesterol/phospholipids ratio in spermal membranes is observed, but in case of short-term exposure it occurs due to the increase of phospholipids quantity, while in case of long-term influence it is due to decreased cholesterol content.

**Keywords:** electrostatic field, maturation of spermatozooids, spermal membrane.

### Introduction

The extreme increase of male infertility has been reported in last years: it increased by 30%-50%. The evidence on the decrease of parameters of spermogram is furnished in articles, the authors of which analyzed the investigations on male spermatogenic function [Carlson E. *et al.*, 1992; Carlson E., 1994]. According to many researchers this fact is conditioned by appearance of new anthropogenic ecological factors, among which electrostatic fields (ESF) with tensions exceeding the natural background are widely distributed and continuously acting ones.

Data obtained due to our previous morphological and ultrastructural studies show that both short-term and long-term influences of ESF lead to structural and functional changes of rat testes; particularly, they change the activity of Leydig's cells, which can result in changes of testosterone secretion and, therefore, in abnormalities of spermatogenesis [Dovlatyan R. *et al.*, 2004; Artsruni G. *et al.*, 2005].

For understanding the effects of ESF to final maturation of male gametes, it is important to investigate the functional activity of spermatozooids after the influence of these fields. It is known that the complete maturation of mammalian spermatozooids occurs in their epididymis and requires definite testosterone concentrations [Orgebin-Crist M.-C., 2005].

The aim of present study was investigation on the influence of ESF with the tension exceeding the natural background on the process of epididymal maturation of spermatozooids and their structure functional state.

### Materials and Methods

White outbred 12-15 weeks aged male rats (body weight 130-150 g) were used as an object of investigations. The same nutritional, light and thermal conditions were provided for both experimental and control animals. The ESF was created using the condenser type device with controlling parameters of the field [Artsruni G., 1983].

The animals were sacrificed by cervical dislocation after 1-hour and fractional (for 6 hours during 6 days) influences of 200 *kV/m* ESF.

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The testes, caudae epididymis and the blood were used for further analyses.

For the avoidance of circadian rhythms, the slaughter of animals has been done in the same time of day immediately after the influence of field.

The quantitative determination of testosterone in blood serum and homogenates of testes was carried out with the help of enzyme immunoassay method. By 6-12 experimental and control animals were used for each experiment. Immediately after dislocation, blood was incubated in thermostat during 15 min at 37°C and blood serum was extracted.

Tissues of testes were treated by 1M CH<sub>3</sub>COOH (2 ml in a sample) during 15 min at +4°C, then they were homogenized in physiological solution (1mg tissue: 9 ml solution) on ice and centrifuged at 15000×g during 15 min at +4°C. Supernatants and blood serum were kept in the deep freeze at -20°C.

Determinations of testosterone concentration were carried out by the automated spectrophotometer Stat-Fax 303 Plus (USA) using DRG Testosterone ELISA kit (DRG Instruments GmbH, Germany) in 420-450 nm range of wave-length.

The preparation of viable spermatozooids from cauda epididymis was carried out by the accepted method [Gustavo A. et al., 2002]. Directly after dislocation the isolated cauda epididymides were weighed, cut into small tissues, and incubated in Ringer/Tris solution (1mg: 9 ml) at +4°C during 30 min. Cell suspensions were gently filtrated for separating large tissue particles. Then the solution was centrifuged during 10 min at +4°C at 130 × g for precipitation of small tissue particles and at 650×g for sedimentation of intact spermatozooids. Extracted spermatozooids were re-suspended in a specified medium for further studies.

Sperm membranes were prepared with slight modification of the method described by J. Luzio and E. Bailyes [Luzio J., Bailyes E., 1998]. Briefly, previously collected spermatozooids were homogenized on ice in 50 mM Tris/HCl and EDTA (pH 7.4) containing 0.25M sucrose. The homogenate was centrifuged at 1000×g for 10 min at 4°C and the resulting supernatant was kept in deep freezing at -20°C for further studies.

Determinations of the functional state of spermatozooids were carried out based on optical estimation of sperm functional activity using vital dyes [Ericsson R., Ericsson A., 1991]. The essence of the method consists in resazurin reduction assay, which depends on the ability of metabolically active spermatozooids to reduce dark-blue resazurin redox dye to pink resorufin. It is a proven fact that the quantity of reduced dye, consequently the optical density, is in direct proportion to the amount of morphological normal and viable spermatozooids with intact acrosome in suspension, as well as to their motility. Briefly, immediately after the influence of ESF, Ringer/Tris solution (0.11M NaCl, 2 mM KCl, 1.4 mM CaCl<sub>2</sub>, 10 mM Tris, pH 7.2) and dye were added to spermatozooids extracted from cauda epididymis in 1:100 ratio. The obtained compound was incubated in thermostat at 48-50°C for 60 min. Then the suspension was cooled up to room temperature and its optical density was measured at 610 nm wave-length by the spectrophotometer. For each experiment 14 control and 16 experimental animals were used.

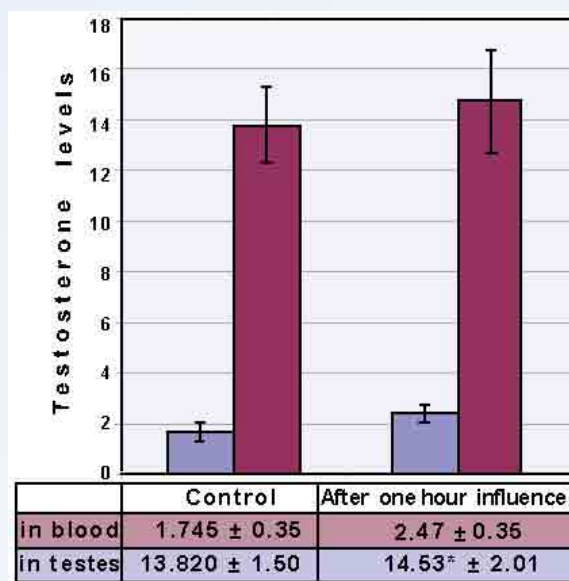
The cholesterol/phospholipids ratio in sperm membranes was determined. Phospholipids were extracted by method of E. Bligh and W. Dyer [Bligh E., Dyer W., 1959], then they were separated using the method of thin-layer chromatography in solvent system, containing light petroleum-diethyl ether-formic acid (30:20:1). The quantity of membrane phospholipids was determined by method of L. Ernster and co-authors [Ernster L. et al., 1950]. The concentration of cholesterol was determined by the method described by A. Courchaine and co-authors [Courchaine A. et al., 1959]. All estimations were done per 1 mg protein. The quantity of protein in membranes was measured by O. Lowry's method [Lowry O. et al., 1951]. Fourteen control and 16 experimental animals were used for each experiment. Every experimental condition was reproduced in four different experiments by three parallels for each point.

**Results**

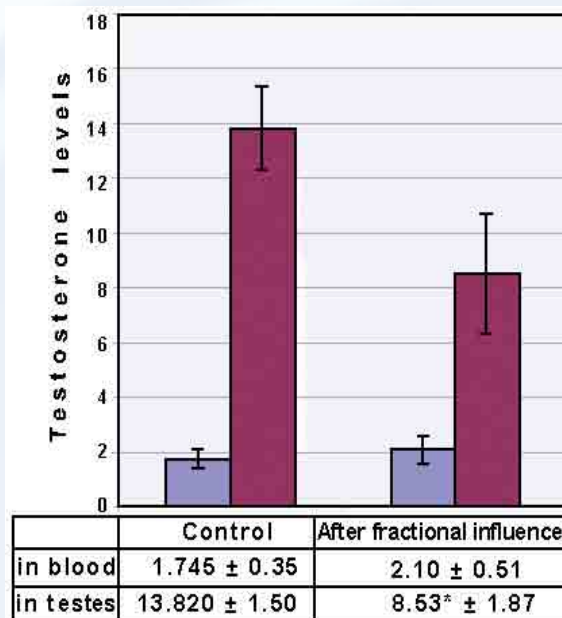
The results of performed hormonal analyses show that after one-hour influence of ESF there was an insignificant (statistically not reliable) increase of testosterone concentration in testes and blood serum by 6.87% and 41.5%, accordingly (Figure 1). After the fractional influence of ESF the reliable decrease of hormone by 38.3% is observed in testes, while in blood serum it does not change (Figure 2).

The spectral analyses carried out using resazurin show that the short-term influence of field leads to the reliable increase of the optical density of spermatozoid-containing solution by 8.57%, but the long-term influence decreases it by 9.71% (Figure 3).

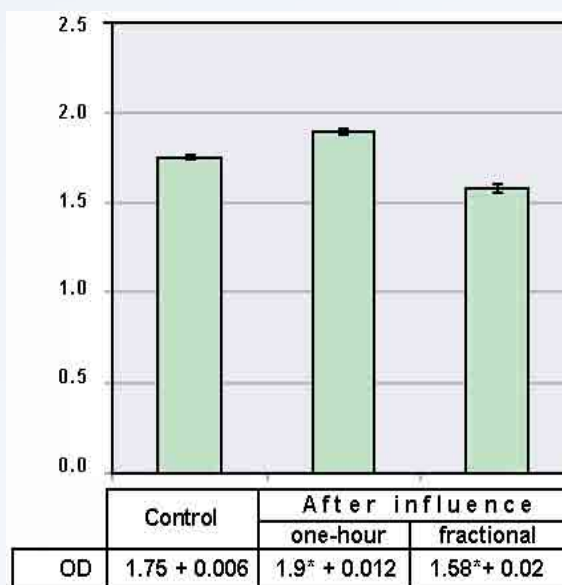
Determinations of the quantity of total phospholipids and cholesterol in spermal membranes isolated from cauda epididymis show that under one-hour ESF influence the quantity of total phospholipids increases 3-fold, while cholesterol levels does not change (Figure 4). After ESF fractional influence 2-fold decrease of cholesterol is observed, while the quantity of total phospholipids does not change (Figure 5).



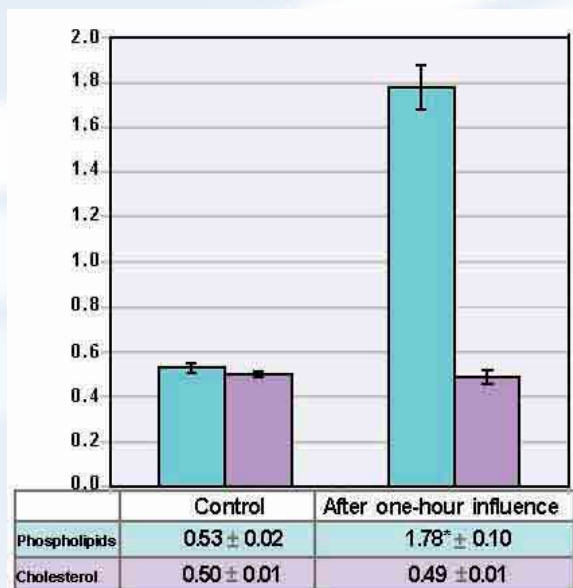
**Figure 1.** Testosterone levels in blood and testes after the one-hour influence of 200 kV/m ESF (ng/ml).  
\* -  $p < 0.01$



**Figure 2.** Testosterone levels in blood and testes after the fractional influence of 200 kV/m ESF (ng/ml).  
\* -  $p < 0.02$

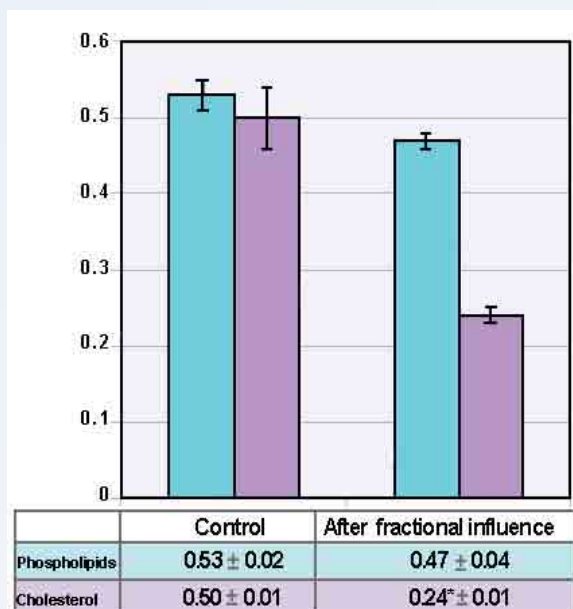


**Figure 3.** The optical densities of spermatozoid-containing solutions after one-hour and fractional influences of 200 kV/m ESF (resazurin reduction test).  
\* -  $p < 0.01$



**Figure 4.** The quantities of cholesterol and total phospholipids in membranes of epididymal spermatozoids after the one-hour influence of 200 kV/m ESF ( $\mu\text{g}/\text{mg}$  proteins).

\* -  $p < 0.01$



**Figure 5.** The quantities of cholesterol and total phospholipids in membranes of epididymal spermatozoids after the fractional influence of 200 kV/m ESF ( $\mu\text{g}/\text{mg}$  proteins).

\* -  $p < 0.01$

## Discussion

The maturation of spermatozoids in epididymis is the finale stage of spermatogenesis. It can be considered as membrane reorganization, during which membranes gain defined lipid composition and surface charge.

It was shown that spermatozoids separated from testes or from initial parts of epididymis do not possess fertilizing capacity and coordinated motility. They mature completely and become viable only in cauda epididymis [Hall J. et al., 1991; Jones R., 1998]. Therefore, spermatozoids separated from cauda epididymis were used as an object for our investigation.

According to literature data, both the hypophysectomy [Orgebin-Crist M.-C., Jonas-Davies J., 1974; Yamamoto M. et al., 1992] and the castration [Orgebin-Crist M.-C., 2005] lead to the atrophy of epididymal epithelium and decrease the weight of epididymis. As a result, spermatozoids lose their viability, and at last, are degenerated. Moreover, the functional state of gametes is restored when the definite quantity of testosterone is added to medium [Turner T. et al., 1985; Yamamoto M. et al., 1992]. This fact demonstrates that the epididymal maturation of spermatozoids is a testosterone-dependent process and requires the certain quantity of this hormone. It is well known that 90% of required hormone flow to the epididymis from testes in protein-binding state and only 10% from the vascular system [Yamamoto M. et al., 1992].

In our previous morphological investigations of testes the increase of spermatozoids quantity was observed in seminiferous tubules after one-hour influence of ESF [Dovlatyan R. et al., 2004]. The results of our present study show that after one-hour influence of field the concentration of testosterone has an uptrend in blood serum and in testes.

The above mentioned information allows us to suggest that observed changes of testosterone quantity are a response of organism to the increased gamete release to the epididymis as a result of ESF influence. This suggestion is based on previous findings [Artsruni G. et al., 1987; 1996], which showed that the effect of electrostatic field resulted in emission of secrets. Hence, we can

suppose that the influence of one-hour ESF can result in increasing the amount of viable spermatozoids in caudae epididymis.

In our earlier morphological and ultrastructural investigations of testes after the fractional influence of field damages of spermatogenetic tissue and a decrease of the amount of morphological normal spermatozoids in seminiferous tubules were observed [Dovlatyan R. et al., 2004; Artsruni G. et al., 2005]. It allowed us to suppose that the amount of gametes would decrease in the epididymis as well.

The results of mentioned hormonal analyses showed that after the fractional influence of ESF the testosterone concentration decreased abruptly, while in blood serum it did not change. Based on the above mentioned literature data, as well as on our findings, we can suggest that testosterone concentration decreases in epididymis as well. Thus, it allows us to expect the decrease of amount of viable spermatozoids in caudae epididymis after the fractional influence of ESF.

Our suggestions were confirmed by results of spectral analyses with the use of resazurin, which were carried out directly after the influence of ESF. According to our findings, the short-term influence of field leads to the reliable increase, while the long-term ESF exposure caused the decrease of optical density of investigated media. This signifies that the amount of mature and viable spermatozoids with intact acrosome increased by 8.57% after one-hour influence of field and decreased by 9.71% after the fractional influence. Taking into consideration that the allowed ratio ranges of optical density in normozoospermic samples is 19.6%, in oligoasthenozoospermic samples 18.9% and in azoospermic samples is 12.0% [Rahman N., Kula K., 1997; Zrimsek P. et al., 2004], then, in spite of low values of observed changes, we can definitely consider reliability of our results.

It is necessary to note that in itself the concentration of spermatozoids can not serve as a parameter of semen fertilizing capacity. For more valuable estimation of ESF effect on the functional activity of spermatozoids it is necessary to

investigate more determinative parameter. According to scientific publications, cholesterol/phospholipids ratio in sperm membranes is such a criterion [Hoshi K. et al., 1990; Jones R., 1998].

It is known that the biological effect of ESF is realized on the border of media with different means of electroconductivity [Artsruni G., 2001], and as the plasmatic membrane is such kind of a system, then in all probability it would be changed under the field influence, to which signified previous research [Artsruni G. et al., 1992, Poghosyan G. et al., 2007].

On the other hand, the results of studies [Artsruni G., 2001; Artsruni G. et al., 2004] showed that total quantity of phospholipids in erythrocyte membranes changed after the short-term influence of ESF, but the ratio of different lipid fractions remained unchanged. Available data [Anderson T., McConnell H., 2002] indicate the changes of cholesterol/phospholipids ratio in membranes under the ESF effect. But as stated above this ratio is an important parameter for estimating the functional state of spermatozoids.

In accordance with literature data, biochemical composition and viscosity of sperm membranes are changed during epididymal maturation [Jones R., 1998]. It is related with the quantities of cholesterol and phospholipids in membranes. The membrane of the head of spermatozoids contains the high quantity of cholesterol, which is known as a regulator of both lipid bilayer viscosity [Hall J. et al., 1991; Haidl G., Opper C., 1997] and the increase of negative surface charge of sperm membranes during epididymal maturation [Langlais J. et al., 1981].

As the membrane lipid composition of spermatozoids isolated from cauda epididymis is equal to the same parameter of mature gametes [Nikolopoulou M. et al., 1985; Rana A.P. et al., 1991; Aveldaño M. I. et al., 1992], then it is possible to suggest that the investigation of membranes of spermatozoids isolated from cauda epididymis, particularly, determination of cholesterol/phospholipids ratio in sperm membranes, will allow us to obtain more complete picture of functional activity changes of epididymal spermatozoids after the influence of ESF.

It is known that cholesterol/phospholipids ratio is a definite value for every species and is a direct characteristic of fertilizing capacity of spermatozooids. Data obtained show that in spermal membranes isolated from cauda epididymis the investigated ratio in norm equals "1", which is in accordance with data available in publications [Poulos A., White I., 1973; Parks J., Hammerstedt R., 1985; Mack S. et al., 1986; Alvarez J., Storey B., 1995]. One-hour influence of ESF increases the quantity of phospholipids, while the cholesterol is not changed compared with control.

The results of study [Artstruni G. et al., 2004] testify that under the same parameters of field the ratio of different lipid fractions stay constant, while the total quantity of lipids is changed. This fact allows us to suppose that in our investigations the ratio of cholesterol to different lipid fractions is changed.

On the other hand, it is a proven fact that the total quantity of sperm membrane phospholipids decreases by 25-48% during epididymal maturation. Some researchers consider that spermatozooids use the fatty acids of phospholipids as an energy source for processes of gamete maturation due to small quantity of glucose [Sinclair S., 2000].

Taking into account that one-hour influence of field decreases the total quantity of phospholipids in spermal membranes thrice and comparing it with the above stated, we may suppose that the influence of investigated factor can bring to changes of energetic balance necessary for epididymal maturation of spermatozooids. This suggestion is circumstantially proved by results of studies [Mkrtychyan S., Artstruni G., 1978; Artstruni G., 2004].

It is common knowledge that for reaching and fertilizing the oocyte spermal membranes must have defined viscosity and fusion, which mostly depends on cholesterol/phospholipids ratio in membranes [Hoshi K. et al., 1990]. The 3-fold decrease of this ratio after the short-term influence of field allows us to conclude that ESF changes the functional activity of spermatozooids. It can be connected with either the activation of phospholipids involvement in spermal membranes or a result of reduced usage of phospholipids as an energetic material.

On the other hand, it may decrease the membrane permeability and fusion, thereby interrupting the process of fertilization, particularly the development of acrosome reaction. However, it cannot affect the quantity, morphology and viability of epididymal spermatozooids.

As shown above, after one-hour influence of ESF the quantity of spermatozooids is increased in epididymis; this latter permits us to suppose that the short effect of field leads to the supplemented release of spermatozooids with reduced membrane viscosity to the epididymis.

After the fractional influence of ESF in spermal membranes the 2-fold decrease of cholesterol quantity is observed in comparison with the control, while the quantity of phospholipids practically is not changed.

According to literature data, the cholesterol/phospholipids ratio in spermal membranes is generated in male sexual tract [Grizard G. et al., 1995]. This hypothesis is also proved by the results of studies, which show that the active synthesis of cholesterol involved in spermal membranes during maturation occurs precisely in epididymis [Parks J., Hammerstedt R. 1985; Hall J. et al., 1991; Yanagimachi R., 1994; Haidl G., Opper C., 1997].

The comparison of these data with our results allows us to suppose that the long-term influence of a field can inhibit the synthesis of cholesterol in epididymis, thereby bringing forth the decrease of cholesterol content in spermal membranes.

Cholesterol is known as a regulator of viscosity of spermal membrane lipid bilayer [Langlais J., Roberts K., 1985; Nikolopoulou M. et al., 1985; Benoff S., 1993]. Thus, it is possible to state that the 2-fold decrease of cholesterol quantity after the fractional effect of ESF decreases membrane viscosity of epididymal spermatozooids, affects their acrosome reaction and processes of fertilization.

On the other hand, in sperm membranes cholesterol exists in the form of sulfite salts [Lalumiere G. et al., 1976] and the increase of negative surface charge of membrane in epididymal maturation is caused by the salts [Langlais J. et al., 1981]. The results of studies [Poghosyan G. et al., 2007] show that both *in vitro* and *in vivo* influ-

ences of ESF change the surface charge of erythrocyte membranes. It is suggested that this may be a result of conformation changes of macromolecules under the direct influence of field or a result of the field effect on metabolic processes occurring in cell and ensuring the surface charge of membranes. Taking into account the duration of field influence and the decrease of cholesterol, we can suppose that in case of ESF fractional influence the metabolic processes regulating cholesterol quantity are damaged in membranes, which in all probability will lead to the changes of gamete surface charge. As a result, the possibility of agglutination of spermatozooids will increase, which is accompanied by a decrease of their functional activity.

Considering that specificity of lipid composition of sperm membranes, particularly, high cholesterol levels, determinates their high fusion, which is necessary for transmission of genetic information [Mack S. et al., 1986; Alvarez J., Storey B., 1995; Chernomordik L. et al., 1995], we can suppose that the observed decrease of cholesterol quantity in spermal membranes of epididymal gametes after the fractional influence of ESF results in the irreversible decrease of functional activity of spermatozooids as a result of which they loose the fertilizing capacity. This conclusion is confirmed by the results of studies [Shafik A., 1992; 1999; 2000], according to which the long-term influence of ESF brings to the temporary male infertility. This fact allowed authors to propose the use of ESF as male contraceptive means.

Data thus obtained show the decrease in number of spermatozooids isolated from the cauda epididymis after the fractional effect of field. It also proves that the investigated influence of field brings to the morphological changes of spermatozooids, which in many respects depend on irre-

versible biochemical changes of sperm membranes. It is well known that all membrane components are involved in processes of spermatogenesis and maturation of gametes [Sanocka D., Kurpisz M., 2004]. It was shown that both quantitative and/or qualitative changes of lipid components decrease the functional activity and permeability of membranes, lead to the inactivation of membrane receptors and enzymes and increase the nonspecific permeability [Jones R. et al., 1979; Esterbauer H. et al., 1988; 1990; Aitken R. et al., 1993].

#### Conclusion

The performed investigations show that one-hour influence of studied field results in statistically non-reliable increase of testosterone concentration and amount of viable spermatozooids, while the fractional influence leads to the decrease of hormone concentration in testes and to the reduced amount of viable spermatozooids in cauda epididymis. In both cases the decrease of cholesterol/phospholipids ratio in spermal membranes is observed, but in case of the short-term influence it occurs due to increasing quantity of phospholipids, while in case of a long-term influence it is a result of the decreased cholesterol content.

Data obtained in our previous studies [Dovlatyan R. et al., 2004; Artsruni G. et al., 2005] and the results of present communication, allow us to suppose that the one-hour influence of ESF to investigated parameters leads to the increase of the amount of spermatozooids in cauda epididymis, but it is accompanied with the decrease of their functional activity. The fractional influence of the same field brings to the decrease of the amount of spermatozooids and results in more deep abnormalities of sperm functional activity. This conclusion correlates with studies, which demonstrated the inhibiting effect of long-term influenced ESF towards male fertility [Shafik A., 1992; 1999; 2000].

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