

## *Influence of Exogenous Factors on the Maturation Levels of Spermatozoa Chromatin in Sub/Infertile Men Treated with Nutritional Supplement PAPA®*

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**Abstract.** Changes in the hereditary information during the nucleus maturation of the sperm cells can occur under the influence of exogenous or endogenous factors. Exogenous factors could be linked with unfavorable working environment or bad habits. In the recent years nutritional supplements have been used for male infertility treatment. In addition, they can improve the morpho-physiological status of the sperm. The present study aims to investigate the effect of nutritional supplement PAPA on the spermatozoa chromatin maturity level by using an Aniline Blue staining test (aniline blue staining - AB) in sub/infertile men exposed to exogenous factors.. A group of men (n = 88) was examined after their written agreement and a completed questionnaire. The tested group with established diagnoses of asthenoteratozoospermia, oligoasthenoteratozoospermia, astenoteratozoospermia with hypovolemia, oligoasthenoteratozoospermia with hypovolemia took orally PAPA® supplement for three months. All patients were tested for DNA damage by an AB test twice before and after the treatment. The patients in the tested group were divided based on the type of harmful work environment (I-IV) and harmful habits (smoking I-III). The results showed that after the treatment with a nutritional supplement PAPA®, the levels of condensation or maturity of sperm chromatin increased by 16.11%. In addition, connections between the effect of treatment and the conditions of the harmful working environment, as well as the amount of received tobacco by the smokers were found. A better effect of the treatment in the tested group with the absence of harmful working environment and non-smokers was found. Therefore, the lowest treatment efficacy was found in the patients who are chronically exposed to occupationally harmful environment, associated with increased body and scrotal temperature (seated workers in factories, ect., drivers, welders, bakers, founders, heavy physical labour workers). On the other hand, a negative connection between the smoked cigarettes per day and the effect of the food supplement in the tested group of men with harmful habits, such as smoking was established.

**Key words:** male sub/infertility, spermatozoa maturity, aniline blue test, nutritional supplement, working environment, cigarette smoking.

### **Introduction**

Chemical and physical agents in the environment introduced and distributed

through various human activities could affect male fertility (JUREWICZ *et al.*, 2009). In addition, the quality of the sperm

parameters in the elderly men can be influenced by a number of environmental and lifestyle factors (PACEY, 2010). Exposure to chronic occupational factors such as high temperature, irradiation, chemicals, dust, etc. could be also associated with male infertility. In the mature sperm cells that have undergone spermiation, single and double strands of DNA could be detected. According to MARCON & BOISSONNEAULT (2004) harmful effects on the genetic integrity of male gametes can arise from this process. As stated by IRANPOUR (2000), KIM (2013) and AFIFA (2013) Aniline Blue (AB) is considered as one of the indirect cytochemical assessment methods for detecting the abnormalities related to measuring the state of the chromatin structure and nuclear maturity of the sperm. Cytochemical analyses are sensitive, simple and inexpensive as they do not require special instruments such as flow cytometry (KIM, 2013). The AB test is also used to visualize condensation, hence the maturity of the spermatozoa chromatin (AGARWAL, 2009; WONG, 2008). In general, the chromosomes in the nucleus of the spermatozooids are tightly packed in a complex of DNA and protamine, as the somatic histones are being replaced during spermatogenesis (BRAUN, 2001). AB staining allows to detect the lysine residues that appear as excess of histones that remain connected with the sperm DNA (DADOUNE, 1988). KIM (2013) in a preliminary research found a connection between chromatin condensation and impaired male fertility.

It is essential that an accurate and adequate treatment should be given when there is evidence of male infertility. One of the options is the use of oral antioxidants included in various nutritional supplements. In recent years, the use of food additives in the treatment of male infertility has increased. It has been found that they could improve the level of

fertility, sperm production and sperm morpho-physiological state as well (ZINI, 2009; LIU, 2009; ROSS, 2010; ALAHMAR, 2018; GADALLAH, 2018). In order to define the effect of application it is important to study the specific influence of a combination of ingredients in the used dietary supplement. Therefore, it is also important to conduct detailed studies of their effects on sperm indicators, as well as hidden anomalies associated with the state of the nuclear chromatin in the sperm cell. In this regard, the application of nutritional supplements has an essential place to solve the reproductive problems. One of the nutritional supplements is PAPA® which specific effect on sperm DNA anomalies is still little known.

Therefore, the aim of the present research is to study in details the effects of PAPA® nutritional supplement on the level of condensation and hence, the maturation level of sperm chromatin by using the AB staining test in sub/infertile men who has bad habits and are exposed to harmful professional working environment.

## **Materials and methods**

### *Experimental setup*

In the present study, a group of men (n=88, 24-51 years old) who agreed to participate in the present study filled out a questionnaire. They are current patients at the clinic of Urology, University Hospital "St. George", Plovdiv, Bulgaria, as well as the andrological laboratory at *In-Vitro* Center, "Selena" hospital, Plovdiv, Bulgaria. The patients were diagnosed by routine performed spermogram (semen analysis) with the use of computer-analysis software, CASA (Computer assisted sperm analyzer, Microoptic). The tested group included 88 patients. They received nutritional supplement, after confirmed sub/infertility with diagnoses asthenoteratozoospermia (AT), oligoasthenoteratozoospermia (OAT), aesthenoteratozoospermia with Hypovolemia

(ATH), oligoasthenoteratozoospermia with Hypovolemia (OATN). The patients from the tested group were divided according to: professional nature of the harmful working environment (I-IV) and harmful habits - smoking (I-III). Table 1 shows the number of tested men and percentage ratio for the individual groups. The tested group excluded men with proven varicocele, urogenital infections, azoospermia and aspermia.

PAPA® nutritional supplement was purchased from the company producer Vital concept Ltd, Sofia, Bulgaria. The men from the tested group underwent a three-month course of treatment during which they did not receive other dietary supplements and medications. The daily dosage was 2 capsules of PAPA, containing L-carnitine (469 mg), L-arginine (280 mg), Coenzyme Q10 (16.0 mg) (CoQ10), Vitamin E (112.8 mg), Vitamin B9 (folic acid) (800 µg) Glutathione reductase (80 mg), Selenium (26.4 µg), Taurine (20 mg) and Fructose (50 mg).

#### *Aniline Blue test*

Sperm cells were stained with Aniline Blue-Eosin according to [WONG \(2002\)](#) and [PARK \(2013\)](#) with some modifications. The test was performed twice – before and after treatment. Slides were prepared using 10 µl of semen sample. Five slides were prepared for each patient. They were air-dried, fixed in 4% formalin for 5 minutes at room temperature, then washed at water bath and stained in 5% Aniline Blue (Sigma-Aldrich Co., USA) dissolved in 4% acetic acid (pH =3.5). The slides were afterwards washed with water and stained in 0.5% eosin (Merck, Germany) for 1 minute. They were rinsed and dried in room temperature air. A number of 200 spermatozoa were observed under a light microscope, using a

lens with X100 magnification and evaluated in different areas of the slide (1000 in total per patient). The underdeveloped (immature) sperm cells were colored in dark blue with contrast coloration of eosin as the normal or mature sperm cells were dyed in light blue (reference values –  $\geq 70\%$  normal cells).

Statistical analyzes were performed with a statistics software for descriptive statistics (STATISTICA version 7.0 for Windows, Stat Soft, 2004). The results were expressed as an average value. The percentage difference was after treatment. Significant differences before and after treatment were established by usage of Student's t-test, with  $p < 0.05$  being considered a statistically significant.

#### **Results**

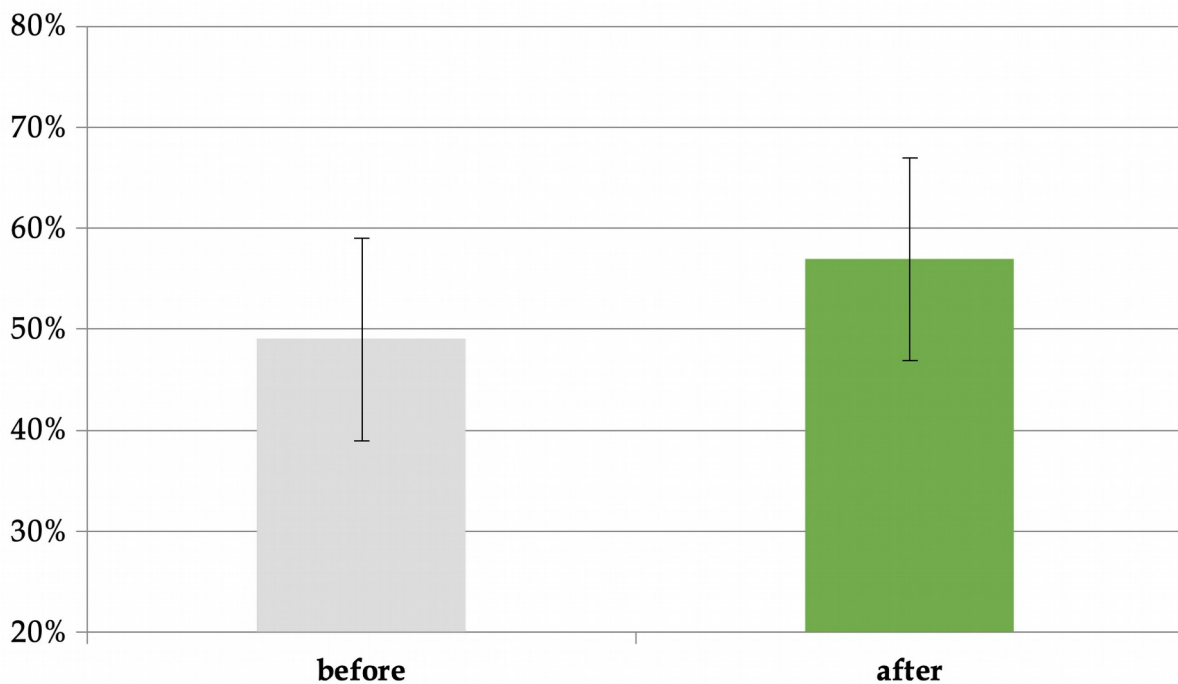
Before the treatment with PAPA in the tested group, the AB test showed high levels of abnormal spermatozoa in 98.86% of the patients.

After the nutritional supplement intake by the men in the tested group the mean values showed a generally positive effect of the therapy with significant difference at ( $p < 0.05$ ). We determined that the levels of maturity of the sperm cell chromatin after the treatment increased with a total percentage change of 16.11% (Fig. 1).

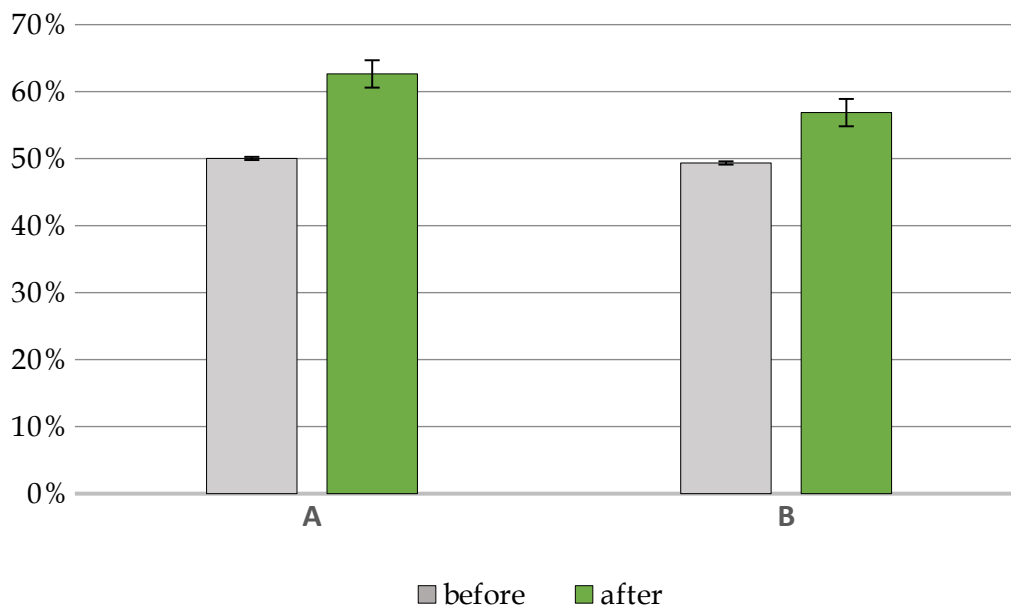
When analysing the results before and after the treatment of the patients who did not report harmful working environment (A) and those who work in such (B) (we found statistically significant differences in both groups patients (A and B) ( $p < 0.05$ ) (Fig. 2). Along with this, the percentage change after treatment in the patients without harmful environment (25.24%) was higher than that in patients with unfavourable working environment (15.23%).

**Table 1.** Number and percentage of patients in the tested group - harmful working environment, harmful habit - smoking and diagnosis.

Patient group	n = 88	%
<b>Harmful working environment:</b>		
I - Work in vapors, dust, gas, oil, paint, lacquers, plastics, metals	30	34.09
II - Working in a sitting position	7	7.95
III - Heavy physical labour	7	7.95
IV - Driver at low and high temperature	20	22.73
Absence of a harmful environment?	24	27.27
<b>Group with harmful habits:</b>		
I - 10 Cigarettes per day	21	23.86
II - 20 Cigarettes per day	26	29.55
III - More than 20 cigarettes per day	5	6.82
Non-smokers	36	40.91
<b>Group with diagnosis</b>		
AT	36	40.91
OAT	22	32.95
ATH	13	14.77
OATH	10	11.36



**Fig. 1.** Results for the spermatozoa chromatin maturity before and after the treatment with nutritional supplement PAPA® for all patients ( $p < 0.05$  or  $p = 0.00002$ ).



**Fig. 2.** Results of the AB staining test for the patients with normal spermatozoa before and after the treatment with nutritional supplement PAPA® in dependence on the presence or absence of harmful working environment: A - absence of any harmful working environment ( $p = 0.01$ ); B - presence of harmful working environment ( $p = 0.0001$ ).

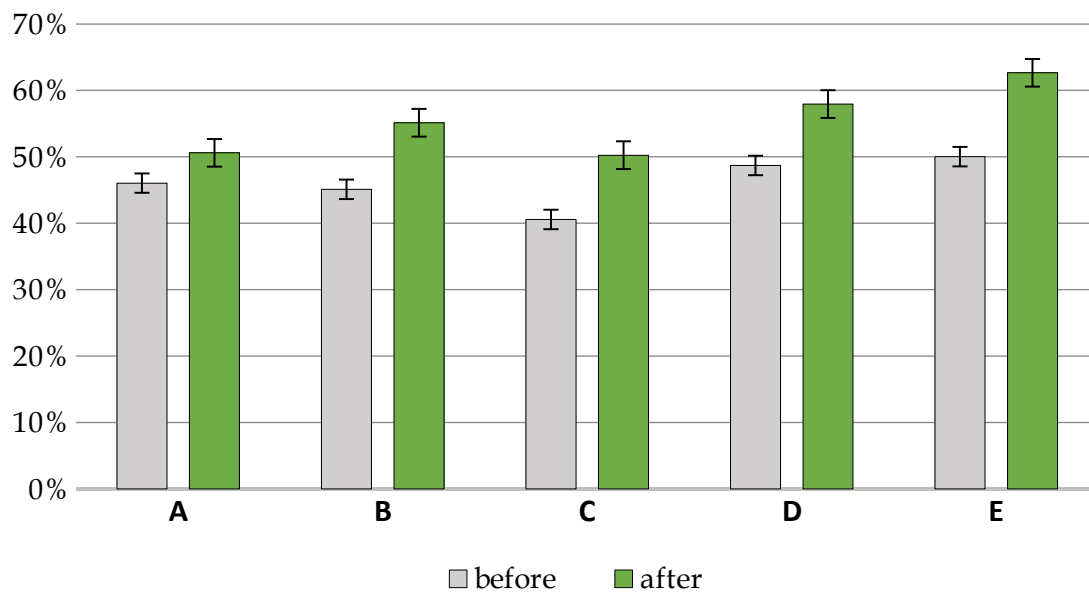
The results for patient groups exposed to different professional harmful working environments are presented in Fig.3. When analysing the results of AB staining, we found statistically significant results in all groups ( $p < 0.05$ ). The highest efficacy of the treatment, of the groups with harmful working environment, was found in group C (a paints, oils, fumes, plastics, dust environment), with the change after treatment being 23.83%. In the other groups the effect of the treatment was lower. Along with this, the percentage change after treatment in the group without harmful work environment was the highest (25.24%). Significant differences ( $p < 0.05$ ) were demonstrated between group of non-smokers (A) (non-smokers) and group of patients who smoke a pack of cigarettes per day (C) ( $p=0.007$ ). The percent change after treatment with PAPA was highest in the non-smokers (16.37%). There was a tendency

towards decreasing the positive effect of PAPA in the group of smokers with increasing the number of smoked cigarettes per day (Fig. 4).

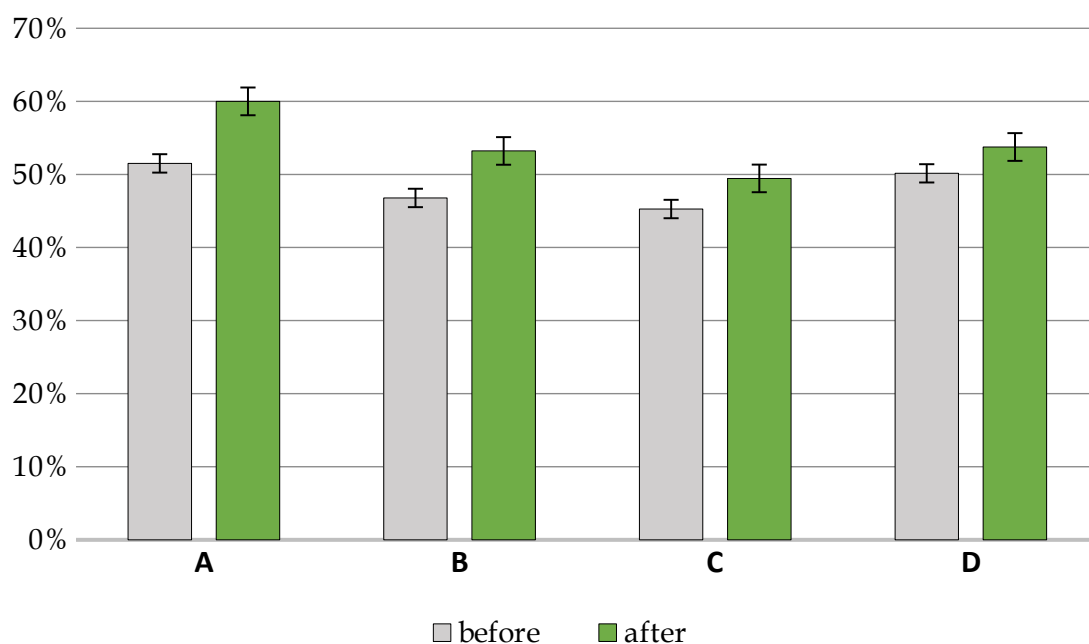
### Discussion

Condensation of chromatin is an important parameter in assessing male fertility, independent of conventional sperm parameters (KIM *et al.*, 2013). There is still insufficient data on the impact of antioxidants on the maturity status of the sperm chromatin, as well as its association with some exogenous factors. For this purpose, we applied a test with AB staining, one of the high sensitivity cytochemical methods, relatively inexpensive and easy to apply in practice.

High levels of abnormal sperm were detected prior to food supplement administration in 98.86% of patients in the test group.



**Fig. 3.** Results of the AB staining test for level of normal spermatozoa in the patients before and after the treatment with nutritional supplement PAPA®: A - seated workers ( $p = 0.017$ ); B - drivers at high temperature ( $p = 0.016$ ); C - workers in paints, oils, plastics, dust, etc. environment ( $p = 0.002$ ); D - heavy physical labour workers ( $p = 0.05$ ); E - patients who reported no harmful working environment ( $p = 0.01$ ).



**Fig. 4.** Results before and after the treatment with PAPA® nutritional supplement for the group of patients with a harmful habit: A - non-smokers; B - patients who smoke 10 cigarettes per day; C - patients who smoke 20 cigarettes per day; D - patients who smoke over 20 cigarettes per day. Total post - treatment percentage change: A -16.37%; B -13.76%; C - 9.25%; D - 7.18%.

Similarly to [HAMMADEH et al. \(2001\)](#) we also found a significant difference in the level of maturity or condensation of sperm cell chromatin by using AB staining test between the trial and control group of patients (healthy donors).

Other authors have also established the abnormalities in sperm chromatin in a sub/infertile men ([MOSKOVITSEV et al., 2009](#); [EVENSON, 2016](#); [AHMADI et al., 2016](#), [YVES et al., 2017](#); [AGARWAL et al., 2017](#); [KÜÇÜK, 2018](#)). Low levels of chromatin condensation and damaged DNA integrity were reported in men from couples with spontaneous abortions when compared to fertile men without such a medical history ([TALEBLI, 2012](#)). According to [AFIFA et al. \(2013\)](#) the integrity of sperm chromatin is essential for successful fertilization, embryonic development and normal pregnancy. We assume the impairment found in the mature sperm may be one reason for the sub/infertility in the patients and these results could be also linked with failures to achieve fertilization.

After of the intake of PAPA®nutritional supplement, the spermatozoa chromatin maturity in the test group increased by 16.11%, suggesting an increase in the male fertility. Moreover, in some patients, the level of maturity became close to the reference/control values. Other authors who have performed previous and similar studies also proved a positive effect on the sperm quality and an increase in the pregnancy rate after using oral antioxidants ([ROSS, 2010](#)). The percentage of positive effect in the experimental group varied between the tested patients, suggesting probably individual specificity. The causes are not clear, but are most likely complex from endogenous and exogenous nature. It is difficult to assess this variability, but in our point of view, an important role for the effects of treatment with the nutritional supplement PAPA® in each and every single patient the state of the antioxidant defence system plays a significant role. This hypothesis was presented earlier by

[WALCZAK-JEDRZEJOWSKA et al. \(2013\)](#). According to the authors a deficit of one of the ingredients in the organism could lead to a reduction of total plasma antioxidant capacity. Along with [SILVER \(2005\)](#), we assume that the oxidative stress affects the function of sperm, which is also an important factor in the aetiology of male infertility. In his analysis [GADALLAH \(2018\)](#) highlighted its importance as an object of interest. The negative impact of oxidative stress is directly related to the increase in reactive oxygen species (reactive oxygen species, ROS), thereby disturbing the natural balance between their production and antioxidant activators in the male reproductive organs. Thus, oxidative stress is seen as a potential cause of male infertility.

On the other hand, the treatment effect depends on the influence of the individual ingredients, quantity and their combination in the nutritional supplement, as well as their interactions i.e synergistic and antagonistic. In previous studies, many authors highlighted the role of diet in male infertility and also that a combined therapy can contribute to improving sperm quality (see [WONG et al., 2000](#); [ROLF & NIESCHLAG, 2001](#); [AL-SHAMMARI et al., 2017](#); [GADALLAH, 2018](#)).

Another important point concerning the effect of the dietary supplement PAPA®, according to us, is the influence of additional exogenous factors, such as work at high and low temperatures, work in a seated position (drivers), heavy physical labour workers, chemicals, dust, smoking, etc. They can also provoke the production of ROS which causes tissue damage, DNA damage and prevent the ability of sperm to function normally. Subjection to the chronic impact of harmful exogenous factors confines the possibilities of antioxidants in the supplement to counteract and offset their negative effect. That is probably why when grouping the patients in the tested group we received various results. Following the results according to the nature of the professional working environment, the

highest rate of change after treatment (23.83%) was in the group working with paints, oils, fumes, plastics. We assume that in the other groups: working seated, drivers, higher temperature during work (welders, bakers, founders), in hard physical labour, the body temperature, and especially the scrotal temperature, is retained for longer time higher. This is essential for the normal course of spermatogenesis and especially for packing the chromatin during the spermatozoon process. It is well-known that the testes are more sensitive to heat and ionizing radiation than any other organ of the body (BONDE *et al.*, 1996). The authors discuss that the specific exposures of the work environment affects the function of the testes. We agree with them that the studies of the impact of the environment on human health is often the most informative and accurate when performed at work, where the exposure can be high and also easily documented. HAMILTON *et al.*, (2016) consider that the heat exposure at work is a significant risk factor for male infertility. Previous studies also found a similar relationship (THONNEAU *et al.*, 1998). Therefore, we think that keeping relatively high body temperature in our patients has a negative impact on the effectiveness of the applied dietary supplement and therefore, positive results in these groups are at lower and values. This is confirmed by the fact that the patients who reported no harmful professional working environment showed the highest effectiveness of treatment with the nutritional supplement PAPA® (25.24%).

Similar dependence was also found in the analysis of the results of groups of patients who smoke. The positive effect varied by values of the individual patients. The highest statistically significant differences and the greatest effect of the treatment we found in the nonsmokers group compared to smokers. We assume that the absence of toxic effect of nicotine in the body plays an important role in the effectiveness of the ingredients of the applied nutritional supplement. The

observed trend that the effect of treatment in smokers decreases with the increase in the daily amount of smoked cigarettes implies an inverse relation between the received quantity of nicotine and the effect of the supplement. We observed a relation between the high levels of damaged with histones sperm cells and smoking as a harmful habit. Previous studies have also established a relationship between smoking and the reduced integrity of DNA of the spermatozoa and nuclear maturation (NIU *et al.*, 2010). According to the study of AVI *et al.* (2015) cigarette smoke causes an increase in ROS, which leads to oxidative stress, which has negative effects on the parameters of sperm and damages the function of the sperm cells, thus reducing male fertility. We support the opinion of FRAGA *et al.* (1996), that smoking and low levels of antioxidants increases oxidative DNA damage in sperm. Some authors even suspect that the father's smoking habit may affect and cause mutations in sperm that lead to cancer, birth defects and genetic diseases in offspring. This reinforces the role of smoking for the occurrence of sperm anomalies and decreased male fertility. Thus, we consider that one of the reasons for deterioration in chromatin maturity and conception problems in our patients. We recommended to our patients, if possible, to change the harmful jobs with other with more favorable conditions, with no harmful influence and also to stop smoking, which is particularly important for patients who have a combination of both factors.

### **Conclusions**

Overall, in the patients of the experimental group the intake of nutritional supplement PAPA® for 3 months caused a positive effect as it increased the level of condensation of the nuclear chromatin sperm with 16.11%. In addition, a link was established between the effects of treatment and the conditions of a harmful working environment, as well as harmful habits (smoking). We observed a better effect of



treatment in the absence of harmful working environment and also in the nonsmoker group. However, the efficacy of the nutritional supplement was least in the patients exposed chronically to harmful environment (associated with increased body and scrotal fever; seated, drivers, welders, bakers, founders, heavy physical labour workers). Lastly, in the smokers group, we found an inversely proportional link between the amount of the bioaccumulated nicotine and the effect of the nutritional supplement. Based on the results received we suggest strongly that further research in this particular area is carried out which would help to better solve the problems with sub/infertility in men.

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