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Original Article

Changes in the Level of DNA Fragmentation in Sperm Cells detected by Acridine Orange Test in Men with Sub/infertility Treated with Nutritional Supplement PAPA

Stoil N. Tomov[1], Radoslava St. Stoyanova[2], Pepa K. Atanasova[3], Ivan Y. Dechev[1]

[1]*Department of Urology and General Medicine, Faculty of Medicine, Medical University of Plovdiv, Plovdiv, Bulgaria.*
[2]*Department of Developmental Biology, Faculty of Biology, University of Plovdiv, Plovdiv, Bulgaria.*
[3]*Department of Anatomy, Histology and Embryology, Faculty of Medicine, Medical University of Plovdiv, Plovdiv, Bulgaria*

Corresponding author: Stoil N. Tomov, Department of Urology and General Medicine, Faculty of Medicine, Medical University of Plovdiv, 15A Vassil Aprilov St., Plovdiv, 4000, Bulgaria; E-mail: stoiltomov57012@gmail.com; Tel.: +359 888149714

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Abstract

Background: When diagnosing and treating male infertility it is important to determine whether there are defects in the maturation process of sperm nuclei. Using nutritional supplements can improve the morphological and physiological condition of the spermatozoa. In recent years there has been an increase in the usage of supplements with different compositions which strives to determine the best combination and avoid side effects.

Aim: To study the effect of PAPA nutritional supplement on the levels of DNA fragmentation of sperm cells tested with acridine orange test (single stranded DNA against double stranded DNA) in men with sub/infertility.

Materials and methods: 48 men with confirmed sub/infertility underwent treatment for three months with nutritional supplement PAPA containing 9 micronutrients. The differences in levels of DNA fragmentation were determined with acridine orange test, which was conducted before and after the treatment.

Results: The results were statistically significant ($p<0.001$) showing an increase in the number of green spermatozoa (normal DNA), and a decrease of damaged ones (orange and red). After treatment the level of sperm DNA fragmentation decreased by 10.2%.

Conclusion: Men with confirmed sub/infertility that took nutritional supplement PAPA for three moths showed a decrease in DNA fragmentation levels of 10.2% determined by AO test which implies an improvement of male fertility levels.

Keywords

DNA fragmentation, nutritional supplement, acridine orange, PAPA, human sperm

INTRODUCTION

The structural integrity of human sperm chromatin and DNA is a determining factor of fertility *in vivo*.[1,2] Many studies point out the necessity of researching levels of DNA fragmentation in men with fertilization problems. Some authors imply that the test should be routinely preformed since it can clarify hidden issues and prevent future failed

pregnancies.[2] Furthermore, defects in nucleus maturation can influence the development of the embryo causing bad reproductive results.[3] One method of evaluating DNA fragmentation is by fluorescence microscopy of dye infused cells with acridine orange.[2-4] It is a tricycle aromatic cationic alkaline fluorescence dye (source), a cellular marker for selective labeling of nucleic acids. In addition, it enters the cell easily and attaches to DNA or RNA by intercalation. Application of this method can also improve diagnosis and treatment of male infertility.[4]

The use of nutritional supplements is the least invasive method of treatment that aims to improve the spermatogenesis and morphological and physiological condition of the sperm cells. Several studies show a positive effect of different antioxidants, microelements and vitamins when used for male infertility.[5-8] In recent years the use of supplements with different quantities and qualities of micronutrients has increased in the search of the best combination without any side effects. However, detailed studies on their effectiveness on treating human infertility are still not sufficient. One of them is PAPA, which contains 9 micronutrients and was recently introduced on the market.

The purpose of this research is to study the influence of PAPA nutritional supplement on the levels of DNA fragmentation of sperm cells, tested with acridine orange test (single stranded DNA against double stranded DNA) in men with sub/infertility.

MATERIALS AND METHODS

In total, 48 men, after having given written consent were tested by completing a questionnaire. All men were between 24 to 50 years of age, and were patients of the Urological Clinic at St George University Hospital, Plovdiv, Bulgaria, as well as of the andrological laboratory at *in vitro* center of the Ob-Gyn Selena Hospital, Plovdiv, Bulgaria. They took the supplement after confirmation of sub/infertility by the means of sperm analysis CASA (Computer Assisted Sperm Analyzer, Micro optic, Spain). The diagnoses included asthenoteratozoospermia (AT), asthenoteratozoospermia with hypovolemia (ATH) and oligoasthenoteratozoospermia (OAT).

Nutritional supplement PAPA®

The substance was provided by Vital Concept Ltd, Sofia, Bulgaria. The men from the experimental group underwent treatment of 3 months. They did not take any other nutritional supplements or drugs during this period of time. The daily dose included 2 capsules per day. The two capsules included: L-carnitine (469 mg), L-arginine (280 mg), coenzyme Q10 (16.0 mg), vitamin E (112.8 mg), vitamin B9 (800 μg), glutathione reductase (80 mg), selenium (26.4 μg), taurine (20 mg), fructose (50 mg).

Acridine Orange test (AO)

Afterseparation and liquefaction of the sperm samples, 5 slides per patient were made and left to dry. After that they were fixed in Carnoy solution for at least 3 hours. After rinsing, drying at room temperature and staining for 5 minutes with acridine orange solution (MERCK, USA) (10 ml 1% AO in distilled water is added to 40 ml 0.1 M citric acid and 2.5 ml 0.3 M $Na_2HP0_47H_2O$).[5] After staining, the glass slides were washed in distilled water and, while wet, were covered with cover slips. The slides were analyzed under ×100 objective lens with immersion oil and photographs were taken with a fluorescent microscope (Leica DM 1000 Fluorescence Filter 13, Germany) with agitation of 450 to 490 nm 1 to 2 hours after staining. For every patient, an overall of 1000 sperm cells were counted (200 per swab). The heads of the sperm cells with normal (double stranded) DNA had a green fluorescent glow and those with denatured or single stranded DNA chain had a red or orange glow, respectively (together with the yellow-orange ones).

Statistical analysis

The results were expressed as mean ± SD. Effect of treatment with nutritional supplement PAPA was assessed by total percentage change in the observed green, orange and red colored sperm cells. Significant differences before and after treatment were confirmed with Student's t-test for paired samples ($p<0.05$). For the statistical analysis the data was processed using STATISTICA version 7.0 for Windows (Stat Soft, 2004, USA).

RESULTS

Over the course of the conducted AO test, sperm cells with normal nucleus and double stranded DNA which had a green glow were observed. The ones with damaged nucleus and/or single stranded had a red/orange glow (**Fig. 1**). Initially 68.8% of the men in the experimental group had high levels of DNA fragmentation in comparison with the reference values and the others had lower levels. The average value in percentage before and after the course of treatment showed an increase in the number of green sperm cells and decrease of the red sperm cells (orange and red), as shown in **Fig. 2**. We confirmed an increase of the green cells and decrease of the red cells, which were statistically significant at $p<0.001$.

The nutritional supplement had a different influence on each patient as the percentage change of the green sperm cells count was variable. After the treatment in 70% of the patients the amount of normal sperm cells increased and in 30% it decreased or did not change significantly (**Fig. 3**). We divided the patients into five groups according to the observed total percentage change of green spermatozoa as follows: over 100%; from 50% to 100%; from 0 to 50%; from

0 to -50%; from -50% to -100% (**Fig. 3**). In the 30% of patients we found that the total percentage change of green sperm cells decreased in the ranges from 0 to -50% (24%) and from -50% to -100% (6%). In the 70% of patients the total percentage change of green sperm cells increased, most pronounced in the group with change from 0 to 50% (34%), followed by those with over 100% (24%) (**Fig. 3**). After treatment the average percentage change of the green spermatozoa in the whole experimental group of patients increased by 10.2%, the orange cells decreased by the 5.2%, whereas the red sperm cells decreased by 5.0% (**Fig. 4**). Consequently the levels of DNA fragmentation decreased by 10.2%.

DISCUSSION

Using the AO test, we found a decrease in the levels of DNA fragmentation after a 3-month course of oral administration of nutritional supplement PAPA by 10.2%. Greco et al.[10] also found a decrease in the fragmentation in spermal DNA after oral administration of antioxidant treatment, which suggests increase in male fertility. The connection between the AO florescent test of the sperm cells and their fertilization ability has been investigated by Hoshi et al.[11], Evenson[12] and others. Initially, more than half of the patients with confirmed abnormal spermogram had high DNA fragmentation levels. This connection is prov-

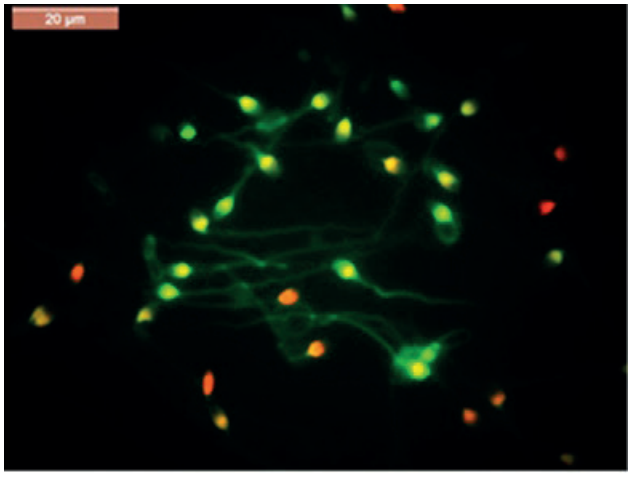

Figure 1. Microphotography of sperm cells of men with sub/infertility. Green glow – normal spermatozoa, orange and yellow-orange – spermatozoa with damaged nuclei; red – spermatozoa with high levels of damaged nuclei. ×600.

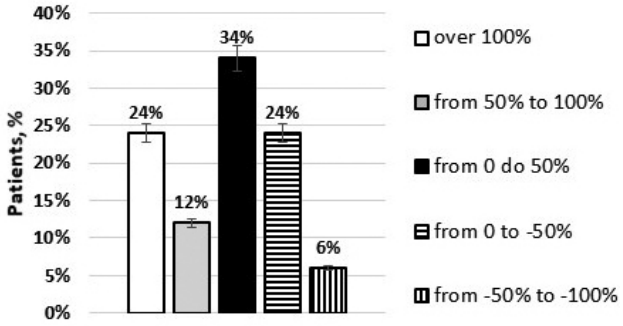

Figure 3. Groups of patients according to the observed total percentage change of green spermatozoa after treatment with nutritional supplement PAPA.

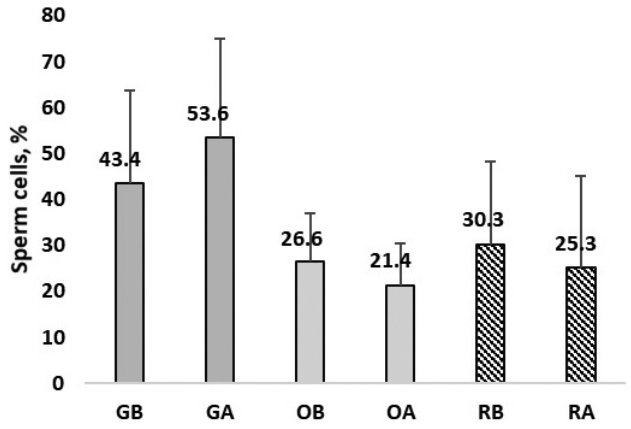

Figure 2. Average values with standard deviation of sperm cells before and after treatment with nutritional supplement PAPA (GB – green before, GA – green after, OB – orange before, OA – orange after, RB – red before, RA – red after treatment).

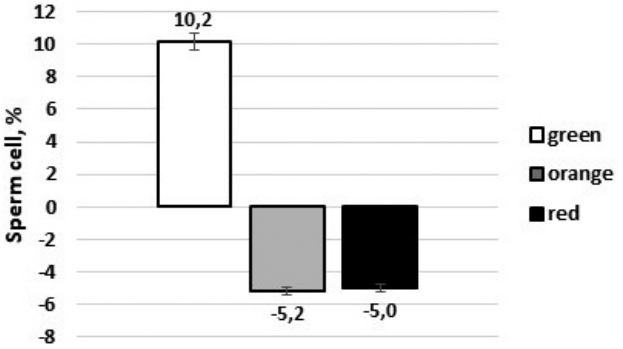

Figure 4. Average percentage change of green, orange and red sperm cells in whole experimental group of patients with sub/infertility after treatment with nutritional supplement PAPA.

en by other authors.[2,4,12-15] Furthermore, the percentage of positive effect was variable in different patients, therefore demonstrating individual specificity. The reason behind this is not yet clear, although it is supposed to be a combination of endogenous and exogenous factors. After the treatment we observed a decrease in both red and orange sperm cells. The percentage decrease of the orange cells was a little bit greater than the percentage decrease red cells (no significant difference), so we consider that PAPA had a positive effect on the spermatozoid integrity of DNA. By proving the spermatogenesis, the nutritional supplement PAPA improves the likelihood for repair and recovery of cell DNA. Abad et al.[16] also studied this improvement of the spermatogenesis. In our case, it is likely that the addition of vitamin B9 had a positive influence, which according to Boxmeer et al.[17] can improve the processes of methylation of phospholipids, proteins, DNA and RNA, and the synthesis and reparation of DNA. It is possible that this recovery is due to interruption of the apoptosis process[18] or lowering the influence of exogenous or endogenous reactive oxygen species (ROS). The inclusion of antioxidants is believed to be responsible for lower ROS levels. Just like Davidova and Yochkova[19], we assume that vitamins E, C, as well as coenzyme Q10 can influence the reduction of ROS, if they are already produced. The combination of all 9 micronutrients in our nutritional supplement increased the amount of healthy sperm cells and decreased the amount of damaged ones. It also lowered the level of DNA fragmentation by 10.2% (p<0.001). However, further detailed research is needed to determine different interconnections and dependencies, which are yet to be represented. Moreover, the experiment was conducted in the course of three months, and it is necessary that the intake is continued with another 3 months. Overall, after application of nutritional supplement PAPA, five of the couples achieved pregnancy without the use of assisted reproductive technologies.

CONCLUSION

Men with proven sub/infertility (AT, ATH and OAT) who took nutritional supplement PAPA for three months had a lower DNA fragmentation levels by 10.2%, which was conducted by AO test and suggests improvement of male fertility. Thus, it is advised that patients continue to take the nutritional supplement.

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Изменения уровня ДНК в фрагментации сперматозоидов, обнаруженные при окрашивании акридиновым оранжевым красителем у мужчин с суб / бесплодием, получавших диетическую добавку PAPA

Стоил Н. Томов[1], Радослава Ст. Стоянова[2], Пепа К. Атанасова[3], Иван Я. Дечев[1]

[1]*Кафедра урологии и общей медицины, Факультет медицины, Медицинский университет - Пловдив, Пловдив, Болгария*
[2]*Кафедра биологии развития, Пловдивский университет „Паисий Хилендарски", Пловдив, Болгария*
[3]*Кафедра анатомии, гистологии и эмбриологии, Факультет медицины, Медицинский университет - Пловдив, Пловдив, Болгария*

Адрес для корреспонденции: Стоил Н. Томов, Кафедра урологии и общей медицины, Факультет медицины, Медицинский университет – Пловдив, бул „Васил Априлов" № 15А, 4000 Пловдив, Болгария; E-mail: stoiltomov57012@gmail.com; Тел.: +359 888149714

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Абстракт

Введение: При диагностике и лечении мужского бесплодия важно определить, есть ли дефекты в процессе созревания ядер сперматозоидов. Приём пищевых добавок может улучшить морфологическое и физиологическое состояние сперматозоидов. В последние годы наблюдается увеличение использования добавок различного состава, которые стремятся определить наилучшую комбинацию, чтобы избежать побочных эффектов.

Цель: Изучить влияние пищевой добавки PAPA на уровни фрагментации ДНК сперматозоидов, протестированных путём окрашивания акридиновым оранжевым красителем (одноцепочечная ДНК против двухцепочечной ДНК) у мужчин с суб / бесплодием.

Материалы и методы: 48 мужчин с установленным суб / бесплодием провели лечение в течение 3 месяцев с помощью пищевой добавки PAPA, содержащей 9 микроэлементов. Различия в уровнях фрагментации ДНК определяли по окрашиванию акридиновым оранжевым красителем, которое проводили до и после обработки.

Результаты: Результаты были статистически значимыми ($p < 0{,}001$) и показали увеличение количества зелёной спермы (нормальная ДНК) и уменьшение повреждения (оранжевый и красный). После обработки уровни фрагментации ДНК сперматозоидов снизились до 10,2%.

Выводы: У мужчин с установленным суб / бесплодием, которые принимали диетическую добавку PAPA в течение 3 месяцев, наблюдалось снижение уровня фрагментации ДНК до 10,2%, определяемое окрашиванием акридиновым оранжевым красителем, что свидетельствует об улучшении уровня фертильности у мужчин.

Ключевые слова

фрагментация ДНК, пищевая добавка, акридиновый оранжевый краситель, PAPA, человеческая сперма