RESEARCH ARTICLE

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Influence of bioactive substances from *Ascophyllum nodosum*, *Lycium barbarum* and *Tribulus terrestris* on the quality characteristics of human spermatozoa

ABSTRACT

The aim of this study is to determine and compare the influence of the major bioactive substances from *A. nodosum*, *L. barbarum* and *T. terrestris* on total sperm count, percentage of normokinetic sperm and total count of normokinetic sperm during an in vivo experiment. As a result of our study on volunteers, a clear tendency was found for the influence of the tested bioactive components on the three major semen quality parameters. It should be noted that the male volunteers studied belong to the group of couples with infertility problems. An increase in total sperm count by 21.9% is a positive indicator to safely conclude that Seanergix ingredients do have an effect on spermatogenesis. Moreover, an increase of 27.5% was established on the proportion of normokinetic sperm in the semen samples. The results of the DNA fragmentation study show the positive effect of the tested bioactive components after 90 days of administration, with an overall decrease in the percentage of DNA fragmentation by an average of 6%.

Key words: *A. nodosum*, *L. barbarum*, *T. terrestris*, spermatogenesis, sperm count, DNA fragmentation, fertility

Introduction

Infertility is a global disease that affects about 15% of all couples of reproductive age. This amounts to 60-80 million couples worldwide (WHO, 2010). However, long-term fertility analysis is difficult to perform due to the fact that more and more couples are delaying or not planning to have children (Mascarenhas et al., 2012). The problem of fertility affects, with equal frequency, both men and women (Templeton, 1992; Sharlip et al., 2002; Abid et al. 2008). Global studies analyzing male sperm have shown that its quality is decreasing, possibly reflecting a worldwide decline in fertility (Rolland et al., 2013; Aitken et al., 2013). There are various causes of male infertility such as varicocele, cryptochordism, cystic fibrosis, infections and tumors (Agarwal et al., 2008). There are also various risk factors that indirectly contribute to infertility, such as smoking, inflammatory diseases and medication intake (Afzelius et al., 1975; Anderson & Williamson, 1990; Tournaye & Cohlen, 2012). Causes and risk factors for male fertility often lead to increased oxidative stress in the semen (Agarwal et al., 2008). For these and other reasons, it is clear that oxidative stress is one of the important factors in male fertility.

Subfertile men often exhibit increased levels of reactive oxygen species (ROS) and/or reduced antioxidant capacity in seminal fluid and in sperm (Fujii et al., 2003). Sperm are particularly vulnerable to oxidative stress not only because of their high content of polyunsaturated fatty acids but also due to inherent defects in the intracellular enzymatic antioxidant defense and limited capacity to repair DNA (Gharagozloo, & Aitken, 2011). Fortunately, enzymatic and non-enzymatic antioxidant molecules are contained in the reproductive tract, including the epididymal and seminal plasma, which work together to protect sperm against a number of toxic oxygen metabolites. Since oxidative stress and sperm DNA damage have been identified as important factors for male infertility and achieving a healthy pregnancy, there is a clear rationale for the treatment of subfertility in men with antioxidants (Gharagozloo & Aitken, 2011). Many aspects of sperm function depend on proper redox signaling, so the different physiological properties of sperm make them excessively sensitive to oxidative stress. Because most antioxidant systems are intracellular, the small volume of cytoplasm in sperm does not provide them with sufficient endogenous antioxidant protection (Zini et al., 1993). Sperm contains very little glutathione compared to somatic cells (Li, 1975; Evenson et al., 1993). The main source of antioxidant protection for sperm is from the seminal fluid surrounding them (Gong et al., 2012). The sperm plasma membrane contains a high concentration of polyunsaturated fatty acids (PUFAs). Due to their unsaturation, PUFAs are particularly sensitive to oxidative

stress, which makes the plasma membrane vulnerable to lipid peroxidation (Wathes et al., 2007). Lipid peroxidation has been used as a fertility marker by sampling thiobarbituric acid reactive substances to measure mainly malonic dialdehyde, a by-product of lipid peroxidation (Kodama et al., 1996). Spermatozoa have virtually no ability to produce de novo proteins by protein synthesis and therefore cannot replace damaged proteins during oxidative stress (Zini et al., 1993).

Any damage to the DNA of the male or female gametes may interrupt the reproductive process. DNA fragmentation of sperm is probably the most common cause of anomaly transmission to offspring and is found in a high percentage of sperm from infertile males. Several hypotheses have been proposed for the molecular mechanism of sperm DNA fragmentation, the most important of which are: apoptosis, abnormal chromatin structure, and the influence of reactive oxygen species. Several studies have shown that DNA fragmentation sperm are capable of fertilizing the oocyte but are associated with a poor quality embryo, blocking blastocyst development and lower pregnancy levels, both naturally and through IUI, IVF or ICSI procedures (Choi et al., 2017). Various studies have shown that oocytes and the embryo retain their ability to repair damaged DNA transmitted by the paternal genome, but it is not yet clear whether all types of damage can be repaired (García-Rodríguez et al., 2019). For example, double-stranded DNA breaks appear to be less repaired than single-stranded breaks and therefore have a greater impact on embryo quality and development. In addition, the capacity of the oocyte to recover DNA depends on factors such as maturity, maternal age, and external factors (Stringer et al., 2018).

The aim of this study is to compare the influence of the major bioactive substances from *A. nodosum*, *L. barbarum* and *T. terrestris* on the total sperm count, percentage of normokinetic sperm and total count of normokinetic sperm during an in vivo experiment.

Materials and Methods

Plant material

The study used a multicomponent product that has been hypothesized to increase sperm concentration and motility with a major content of brown algae, as well as tribulus extract, goji berry and other extracts illustrated below in **Table 1**.

Table 1. *Ingredients of the multicomponent product*

Ingredients:	1 capsule
Brown Seaweed (*Ascophyllum nodosum*) extract	170 mg
Bulgarian Tribulus (*Tribulus terrestris*) extract	110 mg
Korean Ginseng (*Panax ginseng*) extract	20 mg
Wolfberry (*Lycium barbarum*) extract	20 mg
Ginger (*Zingiber officinale*) extract	20 mg
Schisandra (*Schisandra chinensis*) extract	20 mg
Fleeceflower (*Fallopia multiflora*) extract	20 mg

Biological material

The study utilized sperm samples collected in Medical Center Salmanida, Plovdiv. All patients agreed to participate in the study and signed informed consent. Samples were obtained in the period 2017-2018.

Semen analysis

Assessment of sperm quality

Semen analysis is the primary test performed to assess the fertility of men. It involves examining a sample of freshly ejaculated semen under a microscope and conducting a set of tests. Semen analysis is most often done manually, but computerized semen analysis can increase the accuracy of the evaluation when specialized equipment is available. The microscopic examination involves a highly-skilled specialist who places a small amount of semen on a slide and examines it under a microscope. The results of this study are compared with the reference values published by the World Health Organization. The semen analysis test examines a number of semen and sperm characteristics that contain:

Ejaculate volume

Measure the entire amount of semen released during ejaculation.

Sperm Concentration

The concentration of sperm in the ejaculate is determined by counting the number of sperm in each field of view of the microscope. The sperm concentration per ml of ejaculate can then be calculated.

Sperm motility

The ability of sperm to move is a prerequisite for natural conception, and many procedures to assist reproductive technology also depend on the ability of sperm to move and bind to the egg. The semen sample is therefore examined to determine how much of the sperm are motile and to what extent they move. Sperm can be classified as rapidly progressive (i.e., fast-moving), slowly progressive (i.e., moving slowly), non-progressive (moving very slowly) or static (not moving at all).

Sperm morphology

The form of sperm is an important indicator of the ability of sperm to fertilize an egg and thus is an important part of the semen analysis test. Investigated are the shape of the head, the tail and mid-sperm of up to 200 sperm and the data is recorded so that the proportion of morphologically normal sperm in the ejaculate can be calculated.

pH

A pH test is performed to evaluate the acidity of the semen.

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White blood cells

The concentration of white blood cells in semen is evaluated to determine if there are infections in the male genital tract.

Liquefying time

Immediately after ejaculation, semen is a thick mucus-like substance in which the sperm are suspended. Normal semen is liquefied within 15-60 minutes after ejaculation in an incubator at a temperature of 37°.

Sperm vitality

The number of sperm in the suspension that are alive is estimated.

Sperm Chromatin Dispersion Test for DNA Fragmentation (SCD Test)

The SCD determines the ability of sperm chromatin to disperse, which helps to identify DNA fragmentation of sperm (sperm damage). The larger the size of the chromatin dispersion, the less the degree of DNA fragmentation. DNA is contained in the head of the sperm. Fragmentation of spermatic DNA may be a major factor in unexplained infertility and may be associated with chromosomal abnormalities (aneuploidy). During the SCD test, sperm are exposed to hydrochloric acid to denature chromatin and to convert double-stranded DNA into restricted single-stranded DNA. After denaturing, a DNA lysis solution is used to release the DNA strands. These stitches prevent chromatin dissipation in the surrounding areas. The level of DNA fragmentation is measured by estimating the size of the chromatin dispersion using fluorescence or optical microscopy. The extent of DNA damage is inversely proportional to the level of dispersion.

Results and Discussion

For the purpose of the study, a multicomponent product was administered to patients which, according to preliminary hypotheses, increases sperm concentration and motility. A clinical study was conducted with 32 patients who had been taking the supplement for 90 days. Patients were divided into 2 study groups, 12 patients in group 1 and 20 patients in group 2 respectively, according to the time period during which the data was collected. Sperm analysis was performed on day 0 (or before administration), day 60 (2 months after administration) and day 90 (3 months after the administration of the preparation). Sperm data were compared and the effect of drug administration was established. The parameters studied include ejaculatory volume, sperm count in 1 ml of semen, total sperm volume in total ejaculate volume, percentage of normokinetic sperm, percentage of slow sperm, percentage of akinetic sperm, and total sperm counts.

In addition to sperm analysis, data from DNA fragmentation assays of spermatozoa of each patient on day 0 (or before administration), day 60 (2 months after administration) and day 90 (3 months after the administration of the preparation) were compared.

From the data obtained in the study of n=12 patients in group 1, it was clear that there was an improvement in total sperm in 11 out of 12 cases after 90 days of administration of the multicomponent product. The total number of sperm demonstrated an average increase of 19.8% (**Figure 1**).

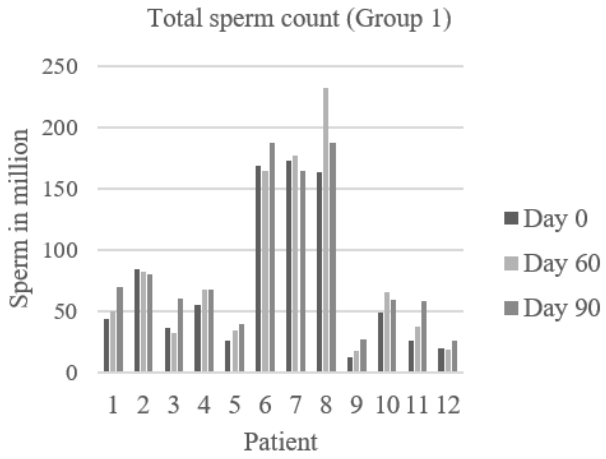

Figure 1. *Total sperm count at day 0 and day 60, day 90 from administration of the supplement with bioactive components (Group 1).*

From the data obtained in the study of n=12 patients in group 1, there was an improvement in the overall percentage of progressively moving sperm in 12 out of 12 cases after 90 days of administration of the multicomponent product. The overall percentage of progressively moving sperm is on average increased by 38.4% (**Figure 2**).

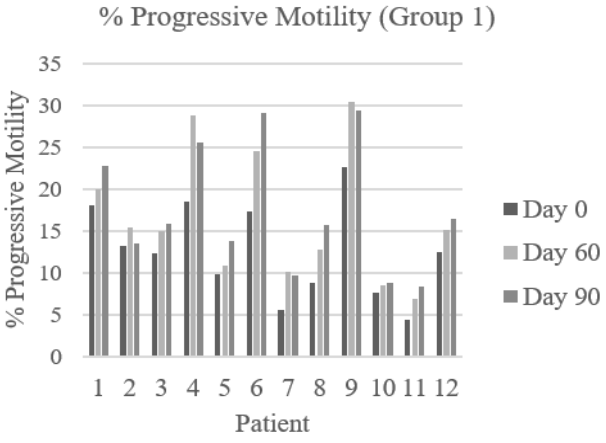

Figure 2. *Percentage of normokinetic sperm at day 0 and day 60, day 90 from administration of the supplement with bioactive components (Group 1).*

From the data obtained in the study of n=12 patients in group 1, it was demonstrated that there was an improvement in the total number of normokinetic sperm in 12 out of 12 cases after 90 days of administration of the multicomponent product. The total number of normokinetic sperm was on average increased by 61.9% (**Figure 3**).

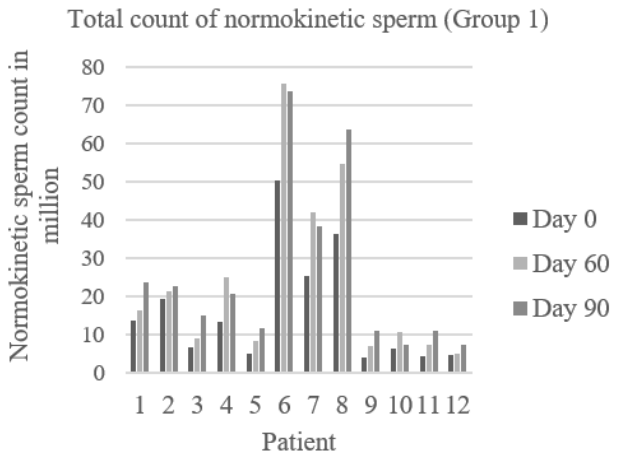

Figure 3. *Total count of normokinetic sperm at day 0 and day 60, day 90 from administration of the supplement with bioactive components (Group 1).*

From the data obtained in the study of n=20 patients in group 2, it was demonstrated that there was an improvement in total sperm count in 17 out of 20 cases after 90 days of administration of the multicomponent product. The total number of sperm had an average increase of 24.1% (**Figure 4**).

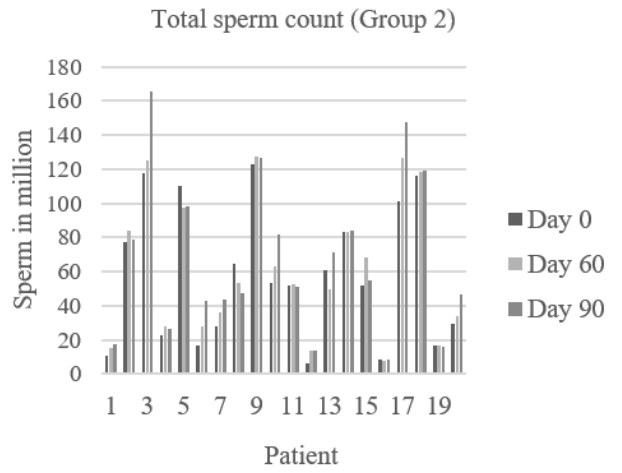

Figure 4. *Total sperm count at day 0 and day 60, day 90 from administration of the supplement with bioactive components (Group 2).*

From the data obtained in the study of n=20 patients in group 2, it was demonstrated that there was an improvement in the overall percentage of progressively moving sperm in 15 out of 20 cases after 90 days of administration of the multicomponent product. The overall percentage of progressively moving sperm is on average 16.7% higher (**Figure 5**).

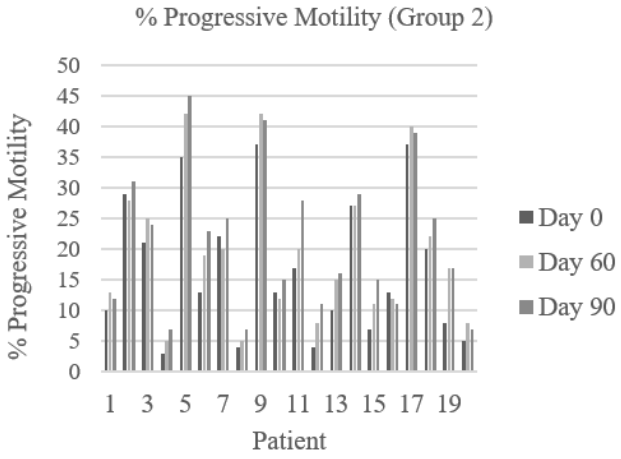

Figure 5. *Percentage of normokinetic sperm at day 0 and day 60, day 90 from administration of the supplement with bioactive components (Group 2).*

From the data obtained in the study of n=20 patients in group 2, it was clear that there was an improvement in the total number of normokinetic sperm in 16 out of 20 cases after 90 days of administration of the multicomponent product. The total number of normokinetic sperm was on average increased by 24.1% (**Figure 6**).

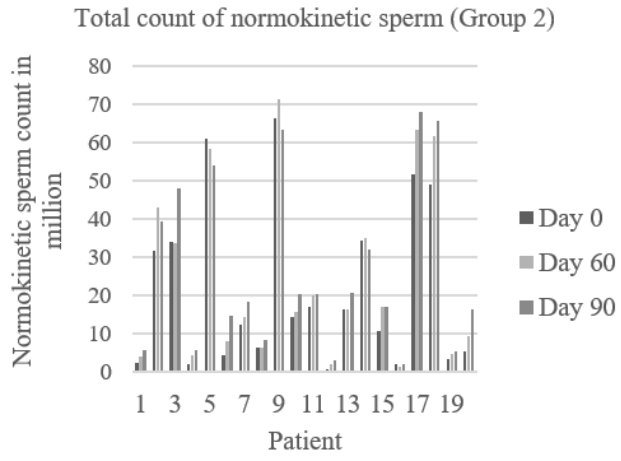

Figure 6. *Total count of normokinetic sperm at day 0 and day 60, day 90 from administration of the supplement with bioactive components (Group 2).*

DNA fragmentation assay

From the data obtained in the study of n=12 patients in group 1, it was clear that there was a decrease in the percentage of sperm with DNA segmentation in 12 out of 12 cases after 90 days of administration of the multicomponent product. The

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total percentage of sperm with DNA fragmentation decreased by 5.5% on average (**Figure 7**).

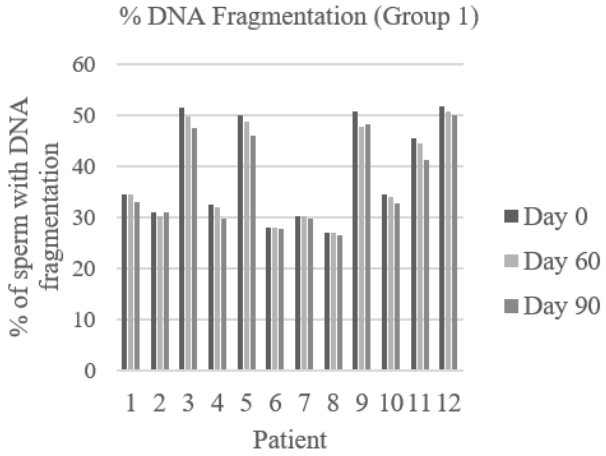

Figure 7. *Percentage of DNA fragmentation at day 0 and day 60, day 90 from administration of the supplement with bioactive components (Group 1).*

From the data obtained in the study of n = 20 patients in group 2, it became clear that there was a decrease in the percentage of sperm with DNA segmentation in 18 out of 20 cases after 90 days of administration of the multicomponent product. The total percentage of sperm with DNA fragmentation decreased by 6.4% on average (**Figure 8**).

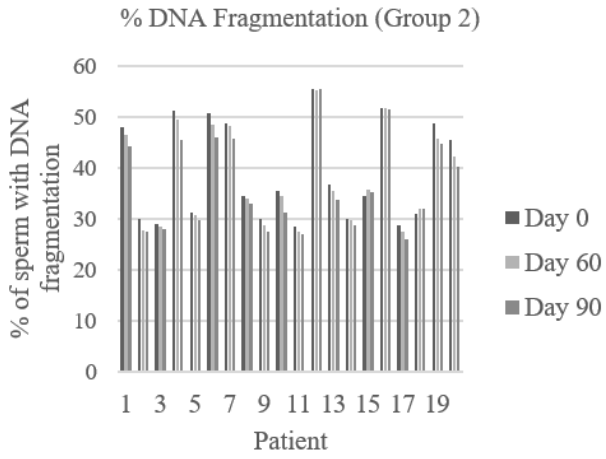

Figure 8. *Percentage of DNA fragmentation at day 0 and day 60, day 90 from administration of the supplement with bioactive components (Group 2).*

Oxidative stress has been studied for four decades. Much progress has been made so far from the descriptive characterization of this process to the outlining of molecular mechanisms that emphasize adaptive responses and target manipulations of expected responses. Oxygen toxicity is an inherent challenge for aerobic life, including sperm, the cells responsible for the reproduction of the species. Oxidative damage to the membranes of sperm, their proteins and DNA is associated with changes in the mechanisms of signal transduction that affect fertility.

Spermatozoa and oocytes have an inherent but limited ability to generate ROS to aid the fertility process. Although there are various protective mechanisms, including antioxidant enzymes, vitamins and biomolecules, a balance between the benefits and risks of ROS and antioxidant substances seems necessary for the survival and function of sperm.

The origins and etiology of elevated ROS in men with suboptimal sperm quality are increasingly clear, presenting many avenues for potential therapy. However, well-designed randomized controlled trials will be required to evaluate the potential of antioxidant systems.

The production of ROS can be beneficial or harmful to living organisms; this also applies to sperm that require low levels of ROS to show their full fertility capacity. Conversely, oxidative stress damages sperm and many other cell types; Excessive levels of ROS are associated with many ailments, including diabetes, cancer, atherosclerosis, and Parkinson's disease.

In recent years, it has been discovered that ROS and ROS-regulated pathways are actively involved in the modification of various cellular processes, ranging from basic metabolism and hormonal signaling to complex processes such as fertilization and development. The aforementioned, along with some biotechnological pathways, would extend ROS-related research in practical directions. Therefore, much remains to be learned about the effects of ROS on biological systems, the adaptive strategies that overcome the ROS attack, and the natural use of ROS in signaling and regulating metabolism.

Conclusion

As a result of the current study, it was determined that the main ingredients of Seanergix, namely *A. nodosum*, *L. barbarum* and *T. terrestris* and their major bioactive components have an impact on the quality characteristics of human spermatozoa, specifically the total sperm count, the percentage of normokinetic sperm, the total count of normokinetic sperm and the level of sperm DNA fragmentation. After 90 days of administration of the supplement to male volunteers with male factor infertility problems, the total sperm count had increased by an average of 21.9%. The proportion of normokinetic sperm in the samples demonstrated an average increase of 27.5%. Moreover, a significant increase of 43% was observed on the total count of normokinetic sperm in the studied samples of semen. Assays on DNA fragmentation indicated a decrease in total levels of sperm DNA fragmentation by a mean of 6%.

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