INCREASED HYALURONAN ACID BINDING ABILITY OF SPERMATOZOA INDICATING A BETTER MATURITY, MORPHOLOGY, AND HIGHER DNA INTEGRITY AFTER MICRONUTRIENT SUPPLEMENTATION

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ABSTRACT

Measuring the hyaluronan-binding ability of spermatozoa is useful in predicting the ability of spermatozoa to fertilise oocytes during *in vitro* fertilisation (IVF). Recent publications discuss an influence of micronutrients on sperm quality. The objective of this paper was to evaluate the effect of a non-prescription nutraceutical containing eight micronutrients on sperm-hyaluronan binding assay (SHBA) values among males with idiopathic sub-/infertility, using an open comparative pilot study. The study took place at the Outpatient Fertility Centre IMI, Vienna, Austria, and involved 67 sub-/infertile males. Sub-/infertile males were invited to participate and take two daily capsules of the active compound for a 3-month period between the first and the follow-up semen analysis. Each capsule contained L-carnitine, L-arginine, zinc, vitamin E, glutathione, selenium, coenzyme Q10 (CoQ10), and folic acid (Profertil®). 40 sub-/infertile men receiving no active treatment served as controls; this was measured by change in SHBA after 3 months. It was found that SHBA values significantly increased after 3 months of treatment with the active compound, from a median baseline value of 56.0% to 74% ($p<0.05$). This represented a 19.7% increase compared to baseline, which was significantly higher than the 2.1% decrease observed in the control group. The rate of subjects displaying an increase in SHBA values after 3 months was significantly higher in the active group (74.6% versus 30.0%, $p=0.0001$), which showed that sub-/infertile men treated with the active micronutrient compound displayed increased SHBA ability. However, more research is necessary to get detailed information on this specific subject.

Keywords: Sperm, micronutrients, sperm DNA integrity, idiopathic male infertility, sub-fertility.

INTRODUCTION

Birth rates in Western countries are decreasing; 10-17% of all couples experience primary or secondary sub-/infertility,[1] defined as the failure to conceive after 1 year of regular, unprotected intercourse with the same partner. Sub-/infertility resulting in permanent childlessness can be a very difficult situation for couples.[2] These couples try to conceive by all possible means, including assisted reproduction (AR), which obviously does not necessarily treat the cause of sub-/infertility.

Factors related to the male partner account for nearly 25-30% of all sub-/infertility causes.[1,3] Several treatable conditions have been identified: hypogonadism, varicocele, gonadotropin deficiency, genital tract infections and obstructions, or sperm

autoimmunity. In about 50% of all sub-/infertile men with seminal abnormalities seeking treatment, no specific cause is found. Despite this, 15% of couples suffering from male factor sub-/infertility have normal sperm parameters, indicating that definitive diagnosis cannot be determined through routine semen analysis.[4]

There is growing evidence highlighting the role of sperm nuclear DNA integrity in male factor sub-/infertility. Reports indicate that a higher amount of DNA damage is associated with a negative effect on fertility potential.[5-7] While sub-/infertile men with poor semen parameters tend to have high levels of sperm DNA damage, increased DNA fragmentation can also be seen in 8% of men with normal semen analysis.[7] Sperm DNA integrity can be estimated by various methods, the sperm-hyaluronan binding assay (SHBA) is one option. Only sperm which have gone through all stages of development are able to recognise hyaluronan as a component of the human zona pellucida. For this reason, DNA integrity is higher in hyaluronan-recognising mature sperm. This is believed to be the main reason for the significant correlation between high SHBA values, good embryonic quality, and lower miscarriage rates. Exposure to environmental and industrial toxins, oxidative stress, smoking, and genetic factors are known to cause sperm DNA fragmentation.[8,9]

Developing an effective treatment for male idiopathic sub/-infertility is not easy. Various agents have been used in an attempt to increase the fertility potential of men with decreased semen quality; nevertheless, studies have rendered heterogeneous results, and the effect of gonadotropins or antioestrogens on pregnancy rates remains controversial.[1] Hence, to date there is still no proven therapy for the improvement of semen quality in this large group of men.[10]

A key factor of major therapeutic interest is nutrition. Most of the essential compounds required for DNA synthesis and spermatogenesis are derived from the diet. Therefore, concentration of required nutrients and other relevant factors may have substantial effects on sperm quality and reproduction.[11,12] A number of nutrients such as trace elements, vitamins, amino acids, and other agents involved in spermatogenesis have been examined and advocated as a way of optimising sperm production and quality. A Cochrane review stated that antioxidant supplementation in sub-/infertile men may improve reproductive outcomes (i.e. live births and pregnancy rates) among couples undergoing ART cycles.[13]

Studies examining the effect of a combination of several of the aforementioned elements and their effect over SHBA are still lacking. Hence, the aim of the present pilot study was to evaluate the effect of a non-prescription nutraceutical containing eight micronutrients on values indicated by the SHBA (maturity, strict morphology, high DNA integrity, and reduced chromosomal aneuploidies) among males with idiopathic sub-/infertility. Micronutrients included in the preparation were L-carnitine, L-arginine, zinc, vitamin E, glutathione, selenium, coenzyme Q10 (CoQ10), and folic acid. The treatment time of 3 months was selected according to the period of 74 days for spermatogenesis and the common interval between first and usual follow-up semen analysis.

MATERIALS AND METHODS

Study Design and Participants

The present open comparative pilot study was performed from January 2007 to October 2010 at the Outpatient Fertility Centre IMI, Vienna, Austria. Men with at least 1 year of sub-/infertility and at least one prior and one recent abnormal semen analysis were invited to participate and take two daily capsules of the proposed nutraceutical for 3 months, after which a follow-up semen analysis was performed (active treatment group). Exclusion criteria were azoospermia, aspermia, varicocele, and recent urogenital infections. Participants in the active treatment group were requested to provide written consent after being informed of the study, its aims, and methodology. Sub-/infertile men attending the Department of Urology of the Medical University of Vienna, Austria, who did not take the active compound during the study period, served as controls. Investigations were approved by the local ethical committee (Vienna) and written consent was obtained from patients.

Preparation (Nutraceutical)

Two capsules of the active compound (PROfertil®) contained: L-carnitine (440 mg), L-arginine (250 mg), zinc (40 mg), vitamin E (120 mg), glutathione (80 mg), selenium (60 µg), CoQ10 (15 mg), and folic acid (800 µg), and were provided by Lenus Pharma GmbH, Vienna, Austria.

Primary Outcome Assessment: SHBA Values

The present study used the SHBA® (Biocoat, Inc., Horsham, PA, USA). The assay kit consists of SHBA® test slides, Cell-Vu® gridded cover slips, and the product instruction. Following 2-3 days of abstinence, semen samples were obtained by masturbation. The samples were stored at 20-30°C for about 30 minutes until liquefaction was complete. Then, 7-10 µl of the obtained semen were pipetted into the centre of the test chamber of the slides. Cell-Vu® gridded cover slips were then installed without entrapping air bubbles. After incubating the slides for about 10 minutes, unbound motile and bound motile sperm cells in the same grid squares were counted. In order to achieve good assay precision the count of bound and unbound sperm cells in each assessed sample was between 100 and 200. This test measures sperm hyaluronan binding (expressed as percentage). The percentage of binding was calculated as 100 x bound motile sperm/(bound motile sperm + unbound motile sperm). A SHBA® score of ≥80% is indicative of normal maturity and physiological function whereas a value of <80% indicates a diminished maturity and physiological function.

Statistical Analysis

Statistical analysis was performed using SPSS version 19 (IBM, Armonk, NY, USA). Data are presented as medians [interquartile ranges], minimum/maximum values, and percentages. The Kolmogorov-Smirnov test was used to determine the normality of data distribution. According to this, differences between groups were analysed with the Mann Whitney test (continuous non parametric data) and the chi-square test (percentages). Changes within each studied group were evaluated with the Wilcoxon rank test. A value of $p<0.05$ was considered as statistically significant.

RESULTS

During the study period a total of 76 eligible sub-/infertile men attending the Fertility Clinic IMI, Vienna, Austria, were enrolled and took the active compound. 9 men withdrew from study participation, leaving 67 subjects who completed 3 months of treatment and provided data for full analysis. The control group included 40 sub-/infertile men. The median age of men taking the active compound was 34 years (minimum/maximum: 18-43 years), whereas in the control group this was 38 years (minimum/maximum: 22-52 years).

SHBA value significantly increased after 3 months of treatment with the active compound, from a median baseline value of 56.0-74.0% ($p<0.05$) (Table 1). This represented a 19.7% increase compared to baseline, which was significantly

Table 1: Sperm hyaluronan binding assay results of the changes between treatment group after 3 months of treatment and control group. The rate of patients displaying an increase in SHBA values is significantly higher in the treatment group.

Parameter	Treatment group n=67	Control group n=40	p value*
Percent of sperm-hyaluronan binding at baseline	56.0 [41.0]	69.5 [23.3]	0.02
Percent of sperm-hyaluronan binding after 3 months	74.0 [21.0] **p=0.0001	64.5 [20.8] **p=0.03	0.01
Median percent change compared to baseline	19.7 [64.8]	-2.1 [7.6]	0.0001
Increase after 3 months *n* (%)	50 (74.6)	12 (30.0)	0.0001
Neutral after 3 months *n* (%)	2 (3.0)	2 (5)	0.99
Decrease after 3 months *n* (%)	15 (22.4)	26 (65.0)	0.0001

Data are presented as medians [interquartile ranges] and frequencies *n* (%).
* p value after comparing groups using the Mann Whitney test or the chi-square test.
** p value when compared to baseline (intragroup comparison) using the Wilcoxon rank test.

higher than the 2.1% decrease observed in the control group (Table 1). The rate of subjects displaying an increase in SHBA values after 3 months was significantly higher in the active group (74.6% versus 30.0%, p=0.0001) (Table 1).

DISCUSSION

The investigated nutrient combination was designed to treat idiopathic male sub-/infertility through the supplementation of several vitamins, enzymes, and trace elements required for optimal sperm cell metabolism, DNA-synthesis during spermatogenesis, proliferation, and anti-oxidative protection. In consideration of their biochemical function, these ingredients are of great significance for male reproduction. A deficiency of these nutrients may result in male fertility disturbances. The studied composition was based on the rationale that each ingredient has been shown to improve sperm factors that may contribute to fertility.

L-carnitine is the energy substrate of spermatozoa. Free L-carnitine is positively correlated with sperm count, motility, and motile sperm density.[14] Although two controlled trials have reported a positive effect of L-carnitine over each of the mentioned parameters,[15,16] a recent study performed on men with idiopathic asthenozoospermia found no significant effect.[17]

Nitric oxide (NO) is beneficial for sperm viability and motility in both fertile and infertile individuals;[18] L-Arginine is the immediate precursor of NO. L-arginine improved sperm motility in infertile men with normal cell counts[19] and displayed a beneficial *in vitro* effect on sperm motility of asthenozoospermic men.[20]

Vitamin E improved sperm motility and enabled fertility in asthenozoospermic men,[21] and also significantly improved the *in vitro* function of human spermatozoa in single studies.[22] In combination with selenium, vitamin E increased sperm motility and normal morphology rates.[23] Selenium is an essential component of the enzyme glutathione peroxidase, and is required for the production of this enzyme when glutathione is supplemented. Testicles contain high selenium concentrations, and sperm quantity and quality are decreased in selenium-deficient humans.[24] Despite this, studies regarding selenium supplementation have rendered contradictory results.[25,26] Zinc is involved in DNA transcription, protein synthesis, testicular development, and sperm maturation, and it is thought to extend functional life span of ejaculated spermatozoa.[27] Low seminal zinc levels have been correlated to decreased fertility potential;[27] zinc supplementation has shown positive effects on sperm counts and other measures.[28] Folic acid is required for DNA synthesis and thus is important for spermatogenesis.[11] Nevertheless, the underlying mechanisms are still unknown. The supplementation of folic acid alone failed to show beneficial effects on sperm concentration in normal and oligo-zoospermic men.[29]

Glutathione plays a key role in protein and DNA synthesis. Lower glutathione levels have been reported in sub-/infertile men and related to abnormal sperm motility and morphology.[30] A positive effect of glutathione supplementation on sperm motility and morphology, and furthermore an oral bioavailability, has been reported.[31,32] CoQ10 is deeply involved in body energy metabolism; 95% of all ATP is converted with the aid of CoQ10.[33] This defines a role for CoQ10 in male infertility, which has been confirmed by increased sperm motility in asthenozoospermic men.[34] Spermatozoa are particularly sensitive to oxidative and electrophilic stress. Moreover, reactive oxygen species have been implicated in both male and female reproductive functions.[35] The oral bioavailability of CoQ10 in the right formulation is documented.[36] Despite this, the role of antioxidants in sperm quality improvement is still controversial, mainly due to the low quality of most studies and to the use of different antioxidants (different combinations, doses, and durations). Pregnancy, the most relevant outcome, was reported in only a few studies.[37]

As extensively described above, each micronutrient alone or in combination have reported positive effects on semen parameters in sub-/infertile men. However, to date no study has reported on the use of a combination of eight of these nutrients (as performed in this pilot study) and its effect on SHBA values. Yagci et al.[38] showed that hyaluronan acid shows a high degree of selectivity for sperm with high DNA integrity. As Breznik et al.[39] stated, the HBA-slide is found to be useful in predicting the ability of spermatozoa to fertilise oocytes in IVF and is helpful in distinguishing semen samples suitable for IVF and intra-cytoplasmic sperm injection. These studies indeed demonstrated that the SHBA can be used for indirect measurement

of sperm DNA integrity. An increase of hyaluronan acid-binding ability means that more motile and morphologically normal sperm with high DNA integrity are binding, which means a higher possibility of achieving an effective fertilisation and a subsequent normal pregnancy. This seems to be an important approach in treating sub-/infertile couples as low DNA integrity values are associated with a lower probability of natural conception, a lower fertilising potential of sperm used in AR techniques, a higher rate of disrupted embryonic development and miscarriages, and a higher probability of diseases in newborns.[40]

The examined preparation was well tolerated by all participants and no adverse reactions appeared. All ingredients have been thoroughly examined for decades and toxicological data show that they exert no negative health effects or potential hazards, even at higher dosages than those used in the present study.

CONCLUSION

We recognise the non-randomised, placebo-controlled design of our study is a limitation, as well as the differences between the controls, such that they did not receive the investigational active compound. However, a double-blind, randomised, placebo-controlled study is currently on its way to support these preliminary results. Despite these limitations, and in light of the fact that therapies for sub-/infertile men are still missing, the investigated compound seems to be a promising therapeutic approach for improving hyaluronic acid-binding ability of spermatozoa in order to enable natural conception among couples with idiopathic male sub-/infertility. In conclusion, hyaluronic acid-binding ability of spermatozoa is improved in sub-/infertile men after treatment with the active micronutrient compound without any adverse effects. More research, however, is warranted in this regard.

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