SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PhD)

New diagnostic and therapeutic options for the fertility potential of human spermatozoa

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1. Introduction

1.1. Historical Aspects of Infertility

Progeny procreation is a genetic instinct, and its failure, the inability to conceive a child, has occupied humanity since the earliest historical times. In ancient times, rites were used, prayers were made to the gods, and sacrifices were presented to facilitate conception, and the ancient Arabs preferred to wear amulets and pendants for this purpose. Even ancient Roman, Persian, and Egyptian physicians clearly described the need for male and female body fluids to meet for procreation, but because they had no specific embryological and microscopic knowledge, the fact of conception itself was unknown. It was generally believed in early medieval Europe that infertility could only be traced back to causes to be found in the female partner, which was often attributed to a spiritual fall. It was typical that if a woman did not become pregnant (regardless of the reason), it was in itself a reason for divorce. Later, in the Age of Enlightenment, it was no longer evident in communities interested in science that women were automatically blamed for the couple's infertility, moreover, in seventeenth-century England, barren men were already treated as a serious health and social problem, as infertility was as reprehensible and degrading to masculinity as impotence. Despite all this, general belief continued to view infertility as a problem for women, but scientific curiosity prompted many researchers to investigate the role of the male side in fertilization. Even before Leeuwenhoek scientifically described the existence of sperm seen under a microscope in 1667, doctors of the age had already described a clear link between ejaculate and pregnancy. Sperm discovered under a microscope were first thought to be parasites, and only some trigger mechanism was attributed to their role in fertilization. Later, a preformed, miniature embryo (homunculus) was thought to be located in the head of the sperm, which begins to grow in the mother's body after the onset of pregnancy (preformationism). In 1824, Jean-Louis Prévost and Jean-Baptiste-André Dumas proved that sperm were necessary for fertilization: in extensive studies of sperm from different types of animals, they found that moving sperm were present in the testicular tissue of sexually mature men and vertebrates and invertebrates too. The need for a scientific analysis of the ejaculate itself and thus for a possible improvement of its quality arose in the early 1900s. Nevertheless, before the 1930s, clinical analysis of sperm was practically not feasible because no standardized data were available against which the fertility of men could have been determined. In the 1930s, more and more studies appeared, in which a reference value could be set both on the basis of biometric analyses and staining procedures, both in morphological and quantitative terms. In

their 1951 study, Macomber and Sanders found a clear correlation between pregnancies and 60 x 106 sperm/mL value. This number became the reference value first used in clinical practice as the normal value for sperm count.

1.2. History of artificial insemination

The first documented human artificial insemination was performed by a surgeon named John Hunter in London in the late 1700s, and the first intervention that resulted in a successful pregnancy was performed in the mid-1800s by an American doctor named J. Marion Sims. The first rabbit born after in vitro fertilization was presented to the public in 1939. The procedure was performed by American biologist Gregory Pincus. The first successful human in vitro fertilization (IVF) resulting in the birth of a healthy living child was performed in 1978 by Steptoe and Edwards on a female patient diagnosed with ovarian obstruction.

During IVF, follicular maturation is monitored by ultrasound after hormonal stimulation. At the appropriate time, the follicle content containing the mature ovum is aspirated with a thin needle – under ultrasound control – and the recovered ovum is placed in a special nutrient solution, and then the specially prepared sperm are also added to the solution. If fertilization or cell division is seen under a microscope, embryos showing regular division are implanted in the uterus on the second or third day after aspiration. IVF is especially useful for low sperm counts in the male partner, endometriosis in the female partner, and – until Intracytoplasmic Sperm Injection (ICSI) was introduced – it was the only solution that could result in pregnancy in bilateral fallopian tube obstruction. The development of ICSI, published by Lanzendorf et al in 1988, was a milestone in the solution of male infertility. This is a revolutionary process because in this case even a single suitable sperm is enough for fertilization. In the procedure, the sperm is aspirated into a thin and sharp pipette, and injected directly into the aspirated egg under microscopic control. The procedure then follows the steps of conventional IVF, i.e. in the case of good fertilization and division, the embryo is placed back into the uterine cavity. This procedure can be performed even in the case of azoospermia (i.e. if the ejaculate does not contain sperm), if a sperm precursor with a degree of maturity suitable for implantation is present in the testis. In this case, a tissue sample is taken from the testis or epididymis using the so-called Testicular Sperm Extraction (TESE) or Micro Epididymal Sperm Aspiration (MESA) techniques – by open surgery or percutaneous needle puncture or the procedure may be performed by microscopic surgical procedure (Microdissection Testicular Sperm Extraction (micro-TESE)).

1.3. Factors affecting sperm function

The production, maturation, and fertility potential of sperm depend on a number of agents inside and outside the body. Disorders of sperm production, maturation, transport, adhesion to and entry into the ovum, and transfer of genetic material can obviously lead to male infertility. Some of these agents cannot be controlled, but some can be treated surgically or by targeted or empirical treatment.

1.3.1. Obesity

Obesity is a major health problem in the Western world. According to data published in February 2016 by the World Health Organization (WHO), the number of overweight adults exceeds 1.9 billion and the number of obese people exceeds 650 million. The effect of obesity on male fertility has been extensively studied in both animal models and humans, and it has been widely accepted as a fact. Several studies support that high Body Mass Index (BMI), along with many other lifestyle factors (smoking, caffeine intake, drug abuse, etc.), also has a negative effect on male fertility.

1.3.2. Varicocele

A varicocele is an abnormal dilation of the pampiniform plexus that causes blood to flow backwards. This may be due to the absence or insufficiency of venous valves or the anatomical difference between the right and left testicular veins. Varicocele can cause testicular pain, lack of testicular development, hypogonadism, and decreased fertility. The background to its negative effect on spermatogenesis is not yet clear, but several factors may play a role. Reflux increases scrotal temperature and intratesticular pressure, causing hypoxia and oxidative stress; toxic substances flow back from the kidneys and adrenal glands and damage the testicles, hormone profile abnormalities occur and the risk of developing anti-sperm antibodies may increase. Varicocele is one of the most common causes of male infertility. It occurs in about 20% of the total adult male population and can reach 40% among infertile men. Moreover, there is an association between damage to sperm production and the severity of varicocele.

1.3.3. Genetic disorders

About 10–15% of male infertility is caused by chromosomal aberrations and gene mutations.
Klinefelter syndrome: the most common sex chromosome abnormality. In these patients, Leydig cell function is impaired, the testes are small, and their phenotype is characterized by androgen deficiency.

In Sertoli cell-only (SCO) syndrome, the testes lack the epithelium that produces germ cells. The lesion may affect one or both testicles.

Y-chromosome microdeletion: One of the most common common genetic causes in the background of male infertility is the presence of microscopically undetectable deletions on the long arm of the Y chromosome. These deletions are most often associated with azoospermia but deletions in certain regions only cause oligozoospermia. It is important to know that the deletion will also be present in the boy's offspring during successful intracytoplasmic injection (ICSI).

In *Kallmann syndrome*, from a urological point of view, hypogonadotropic hypogonadism, testicular maldescent, unilateral renal aplasia may occur.

Cystic fibrosis is an autosomal recessively inherited disease. The mutation occurs in a gene encoding an ion channel (cystic fibrosis transmembrane conductance regulator – CFTR) involved in chloride ion transport. In these patients, developmental abnormalities of the seminal tract may occur.

Other genetic disorders

Without being exhaustive, the *testis expressed 11 (TEX11) gene mutation*, *Prader–Willi syndrome*, *Noonan syndrome*, *androgen insensitivity disorders*, *Robertson translocation*, etc. can be mentioned. These disorders are rare, and for these patients, having a child is usually out of the question either.

1.3.4. Obstruction

In obstructive azoospermia, no sperm are found in the semen. Obstruction of the seminal tract can occur from the testis through the epididymis and the vas deferens to the ejaculatory ducts. TESE is the only option in the treatment of intratesticular obstruction, however, for epididymal obstruction, MESA is recommended. Vasovasostomy is recommended for obstruction at the level of the vas deferens, and transurethral resection of the ejaculatory ducts (TURED) for obstruction of the ejaculatory ducts.

1.3.5. Testicular maldescent

Testicular maldescent is the most common genital abnormality. In undescended testicles, germ cell degeneration begins in the first year of life. In the case of unilateral maldescent, the chances of having a child are almost the same as of those without such a problem (89.7% vs. 93.7%).

However, in the case of bilateral testicular maldescent, spermatogenesis is predominantly impaired, and the fertility of these patients is reduced to 35-53%.

1.3.6. "Idiopathic male infertility"

No clear cause of infertility was found in at least 44% of male infertility. In many of these cases, there may be some form of – even temporary – functional impairment (heat, etc.). Recently, increasing attention has been paid to damage caused by oxidative stress.

1.4. Effect of oxidative stress on sperm fertility potential

Oxidative stress is known to contribute to impaired spermatogenesis because the plasma membrane of sperm contains large amounts of unsaturated fatty acids that are necessary for the acrosome reaction of the cells and the fusion with oocyte membranes. However, the presence of unsaturated fatty acids makes the membrane particularly sensitive to oxidative damage. Sperm that produce high levels of Reactive Oxygen Species (ROS, such as superoxide anion and hydrogen peroxide) are unable to fuse with the ovarian membrane, which leads to male infertility. In addition, white blood cells present in seminal plasma, especially neutrophils, can also be potential ROS producers, and reactive molecules (oxygen radicals, OH) that accumulate in the male reproductive organs can damage the cell membrane of sperm, resulting in significantly reduced fertility. Antioxidant therapy is therefore receiving increasing attention as it may be useful in the treatment of infertility caused by oxidative stress. Antioxidant therapy can improve sperm concentration and motility, and reduce the degree of DNA fragmentation in sperm.

1.5. The role of micronutrients in fertility

Antioxidant vitamins C and E act against ROS, which causes membrane damage to sperm, thereby improving fertility. Several studies have shown that vitamin D has a good effect on male fertility, probably due to improved sperm movement. Vitamin B12 also has an antioxidant and also an inhibitory effect on homocysteine-toxicity. The effectiveness of amino acid therapy is due to the fact that, on the one hand, amino acids are the constituents of structural proteins involved in sperm motility and, on the other hand, bind toxic heavy metal ions, thereby protecting sperm function. Therefore, e.g. arginine to increase sperm motility and the important role of carnitine in sperm maturation processes are also well known. Taurine has been described to have a positive effect on sperm parameters in addition to its antioxidant effect by increasing the secretion of FSH and testosterone. In the absence of zinc, sperm maturation, sperm count,

and motility can also be impaired. Nowadays, several complex preparations are available, which contain in a tablet the beneficial vitamins, amino acids, trace elements, which can be useful supplements for the therapy of male infertility.

1.6. Andrology as a discipline

The study and treatment of male fertility is addressed by andrology, a relatively new discipline among the classical medical disciplines. In its initial period, andrology practically specialized in spermatological examinations, but later it became a complex science of men's health, which includes the examination and treatment of erectile dysfunction, other sexual dysfunctions, disorders caused by male hormonal changes, pharmacological and operative treatment of male infertility and the issue of male contraception; recently, as artificial insemination procedures have become widespread, andrology has become an obligatory prerequisite for them.

1.7. Conventional sperm test parameters and functional tests in andrological examination

Spermatology, being the classical method of examination, is a basic test in the examination of male infertility, but it is not uncommon for men with normal values in conventional sperm analysis to be infertile in practice. In these cases, the causes of infertility cannot be clearly revealed by conventional spermatological examination, and conventional sperm analysis based on this assessment does not necessarily reflect the in vitro or in vivo fertility potential of sperm. In recent decades, intracytoplasmic sperm injection has become an increasingly preferred method for infertile couples for whom a clear cause of infertility has not been established previously. During these procedures, it became clear that conventional sperm parameters did not affect ICSI results, in contrast to molecular and cellular differences that, in turn, could not be detected by basic spermatological tests. For all these reasons, there is a consensus in andrological practice that in many cases more comprehensive sperm function tests should be performed, i.e. additional tests with better predictive values on the pregnancy chances of couples are needed, and that it is important to identify sperm dysfunction at the cellular and molecular levels as accurately as possible. Ideally, ejaculated sperm should have good ability for capacitation, recognize the zona pellucida and be capable of the so called acrosome reaction to be able to fertilize the egg. Tests that evaluate these abilities are called sperm function tests.

1.8. The role of functional tests in modern andrological examination

Although there are tests among the conventional spermatological procedures that provide information on function as well, these tests – e.g. vital staining – mostly provide information only about the viability of the sperm, but if there are living sperm, they do not reveal the cause of male infertility. Functional tests in the modern sense examine the fertilizing ability of sperm in vitro.

1.8.1. Sperm penetration assay

One of the first functional tests developed was the sperm penetration assay (SPA). The assay examines the sperm capacitation ability, the acrosome reaction, the fusion with and penetration through the oolemma, and the ability to decondense in the cytoplasm of hamster oocytes.

1.8.2. Sperm-zona pellucida binding assays

The contact between spermatozoa and zona pellucida (ZP) is extremely critical and such assays provide important information on many features of sperm function. There are several different test methods, but each of them evaluates the tight binding of sperm to ZP as a primary result. It is also important to note that highly predictive values have been demonstrated for successful insemination and in vitro fertilization outcomes.

1.8.3. Hypo-osmotic swelling test

The hypo-osmotic swelling test (HOST) is based on the presence of the intact membrane structure of viable sperm. The membrane of the intact sperm cell plays an important role in the process of fertilization. Under hypo-osmotic conditions, the intracytoplasmic spaces of intact sperm cells swell and their tails twist. Sperm that have an undamaged membrane but are not living are unable to swell in the hypotonic medium. The results of this test correlate with other ejaculate test indicators such as morphology and motility, but information on fertility is inadequate.

1.8.4. DNA fragmentation tests

The integrity of the paternal genome is of great importance for a viable pregnancy. Fragmented DNA in sperm is incompatible with normal embryonic development. DNA damage can occur due to chromatin conversion, apoptosis, or DNA strand breakage during spermatogenesis. Literature data support that post-testicular damage during the passage of sperm through the epididymis, environmental toxins, or ROS-induced fractionation in the genital tract may also play a significant role in sperm DNA fragmentation.

1.8.5. Hyaluronan binding assay (HBA®)

The hyaluronan binding assay (HBA®) is one of the new tests introduced in recent years into the practice of andrology to allow functional examination of sperm; it is simple to perform, but has proven to be very effective prior to planned assisted reproductive interventions. The theoretical basis of HBA® is that hyaluronic acid is present in the human body in almost all tissue types as well as in the female genital tract, e.g. in the cervical mucus or the cumulus oophorus, with which sperm comes into contact. Only mature sperm expressing hyaluronic acid binding receptors (HBRs) are able to bind to the cells of the cumulus composed of a polysaccharide matrix containing hyaluronic acid – similarly to the zona pellucida in vivo – which provides a new opportunity to distinguish between mature and immature cells.

Hyaluronic acid binding sperm selection is a safe and promising sperm selection method to improve the efficiency of intracytoplasmic sperm injection, which led to the introduction of the " Physiological ICSI /Picked for Intracytoplasmic Sperm Injection" (PICSI®, Biocoat Inc., Horsham, PA, USA) method into clinical practice. This selection is also effective in reducing the risk of transmitting numerical chromosomal abnormalities to the offspring; hyaluronic acid shows a high degree of selectivity for sperm with good DNA integrity, and thus the HBA® test also provides information on DNA integrity, which in many cases makes it unnecessary to perform a genetic test. Extensive, well-designed studies found that the rate of loss of pregnancy was significantly higher when pre-procedure HA binding was below 65%, therefore the lower reference limit for hyaluronan binding is considered to be 65%.

2. Aims

Conventional spermatological examinations are cost-effective and quick to perform, but do not provide substantial information for many of today's procedures, while functional tests examine fertility primarily from the perspective of in vitro fertilization. As sperm density is required for treatment strategies and motility affects HA binding capacity, we assume that in everyday andrological practice, the HBA score alone – without knowledge of sperm density and the proportion of progressively moving spermatozoa – does not provide sufficient information to assess male fertility.

In our research group, there was a need to establish a value that takes into account sperm count, movement and also gives a good picture of the capacitation ability of sperm.

It is also important to determine the place of antioxidant treatment in the therapy of male infertility, as well as whether adjuvant therapy is indeed effective, and how its functional efficacy can be measured objectively.

Based on all this, the aims of our first study were as follows:

1.) By further developing the HBA test, introduction of a new index, HB-MaSC (Hyaluron Bound Matured Sperm Count), which takes into account sperm density and motility in addition to HB binding capacity. Consequently, the test and its results combine the elements of conventional and new functional tests.
2.) To measure the effectiveness of oral supplement therapy in the treatment of male infertility using the HBA score and the new index.

The aim of our other study was to investigate the suitability and usability of the new index – HB-MaSC – in andrological patients. In our study, we analysed the effect of one of the most common detrimental factors, obesity, on conventional sperm parameters (sperm concentration and progressive motility) and fertility based on hyaluronan binding, and the applicability of the new index we developed (HB-MaSC) in everyday andrological practice.

3. Patients and methods

To quantify the suitability of the hyaluronic acid binding assay and a new fertility index derived from it in the analysis of parameters such as male fertility and the effectiveness of adjunctive therapy, data from 175 patients over 18 years of age who were infertile for more than 12 months were analysed and evaluated. For every patient, we performed physical and testicular ultrasound examination, microscopic semen analysis (according to the WHO manual for semen analysis, fifth edition, 2010) and HBA test. The study also includes the follow-up of 39 patients of these 175 men, who were checked by microscopic semen analysis and HBA® test after supplement therapy (treated group); the remaining 136 patients had only one examination as they failed to reappear on follow-up examination due to unknown reasons (untreated group); therefore, their data were not included in the comparative statistical analysis. In this study, complex dietary supplements were taken by patients orally during the therapy, which included most of the nutritive factors in proper dose that had been proved to be effective in previous studies and have high antioxidant content: L-carnitine L-tartrate: 148 mg, arginine-hydrochloride: 90.78 mg, Taurine: 125 mg, tocopherol-acetate (75%): 105 mg, vitamin C: 63 mg, magnesium-oxide: 78.20 mg, zinc sulphate monohydrate: 9.61 mg, beta-carotene (20%): 9.45 mg, vitamin D3: 100 000 IU g-1, vitamin B12 (0.1%): 1.31 mg, pyridoxine-hydrochloride: 0.97 mg, coenzyme Q10: 5.25 mg, folic acid: 0.21 mg, sodium-selenite-mannite (Se 0.2%): 9.19 mg (FER14®, distributed by Andromedic Ltd., Debrecen, Hungary). Dietary supplement therapy lasted for a minimum of three and a maximum of 6 months, depending on patient compliance, and the follow-up examination was performed after completion of the oral therapy. No adverse and/or side effects occurred during the follow-up period.

Spermatological examinations were performed after a standard four-day abstinence with a phase-contrast microscope (magnification 200–400×) and a Makler counting chamber under constant conditions (using 10μL of liquefied native semen at room temperature). At least two analyses were performed and the mean of the results was taken into account. Data collection and evaluation were performed according to the standards of the WHO laboratory manual for the examination and processing of human semen, 5th edition (2010). The functional evaluation of sperm was performed using the commercially available HBA® tests (Biocoat Inc., Horsham, PA, USA). The tests were performed according to the manufacturer's recommendations.

We found it practically useful to organize the data from the parameters already in use (sperm density, motility, morphology) and the results of the HBA® test into one index. We call this

index HB-MaSC (Hyaluronan Bound Matured Sperm Count), which stands for the product of the number of spermatozoa in 1 mL semen, the percentage of progressively motile sperm and the percentile of moving spermatozoa binding to hyaluronic acid. Progressivity (PR) was determined according to the criteria of the WHO Handbook (2010) in 10^6/mL.

$$\mathrm{HB-MaSC} = \text{Number of spermatozoa } (10^6/\mathrm{ml}) \times \mathrm{WHO-PR}\ (\%) \times \mathrm{HBA}^{®}\ \text{score}\ (\%)$$

According to World Health Organization (WHO) reference values for fertile men and previous studies on HBA, the lower reference limit for HB-MaSC is 5.6×10^6 / mL (using the WHO values belongs to the "10 Centiles").

Statistical tests were performed using STATISTICA for Windows (StatSoft Hungary Ltd., Budapest, Hungary). T-tests, Mann–Whitney test and Chi-square tests were used in the analysis.

In our other study, we examined the correlation between the number of hyaluronan bound matured sperm count (HB-MaSC) and body mass index (BMI). The study included 72 men who were referred for andrological examination due an infertile relationship that lasted for more than 12 months. The age of the patients in the study ranged from 24 to 43 years (mean age: 33.9). For each patient, we performed BMI calculations, physical and ultrasound testicular examinations, microscopic semen analyses, and HA binding capacity tests. Hyaluronan bound matured sperm count (HB-MaSC) was also calculated. Medically or surgically treatable disorders were part of the exclusion criteria. Patients with clinically relevant varicocele, testicular volume less than 25 cm^3, or other known medical factors such as severe hormonal abnormalities or regular smoking were also excluded. We also excluded patients with teratozoospermia whose normal sperm morphology was below 4%, on the basis of Krüger's strict criteria.

3.1. Body mass index

Although it does not reflect the real percentage of body fat, BMI is accepted and widely used to assess the ideal weight and the extent of deviation from it in clinical research as well as in medical and thus andrological practice. BMI is defined as the weight in kilograms divided by the square of height in meters (kg/m^2), thus measuring BMI is cost-eff ective and easy to perform. For the anthropometric data from which BMI was calculated, height and body weight were measured with a height measure and a scale, respectively.

3.2. Semen analysis

All semen samples were collected in a private room near the laboratory after a 4-day abstinence, by masturbation and ejaculation into a clean plastic container, according to the WHO guidelines. After liquefaction of fresh, untreated samples, the HBA test was performed first, according to the instructions of the manufacturer. Spermatological examinations of the semen samples such as sperm count, motility, morphology, vitality etc. were performed with full respect to and following step-by-step instructions of the "WHO laboratory manual for the examination and processing of human semen 5th edition" using a bright field microscope (magnification 200–400×) and a Makler counting chamber under constant conditions (using a 10μL drop of liquefied native semen at room temperature). Data collection and interpretation were also performed based on the standards of the same WHO manual for semen analysis. Categories of sperm movement were as follows: progressive motility (PR), when the sperm moves actively regardless of speed either linearly or in a large circle; non-progressive motility (NP), when the sperm moves without progression, and immotility (IM), which means no movement.

3.3. Hyaluronan binding assay (HBA®)

The functional evaluation of sperm quality was performed using the commercially available HBA® test (Biocoat Inc, USA). The test was performed according to the manufacturer's recommendations by dropping 7-10μL of native semen onto a hyaluronic acid coated slide and determining the sperm binding rate after 10-15 minutes of incubation at room temperature.

3.4. Hyaluronan bound matured sperm count (HB-MaSC)

Combining the conventional sperm parameters (sperm density, motility, morphology) with the results of the HBA test, the authors formerly described a new fertility index, called Hyaluronan Bound Matured Sperm Count (HB-MaSC), which stands for the product of the number of spermatozoa in 1 ml semen, the percentage of progressively motile sperm and the percentile of moving spermatozoa binding to hyaluronic acid, and which was calculated from the available data.

3.5. Statistical analysis

The relationships between BMI and the outcomes of HB-MaSC, sperm count and progressive motility (WHO-PR, which indicates a value measured in accordance with WHO guidelines) were evaluated using multiple linear regression adjusted for body weight and age. Variables were transformed to improve normality: HB-MaSC and sperm count were square-root transformed, WHO-PR was square transformed, and BMI was natural log transformed. The model contained the first, second, and third power variants of log-BMI, and the first and second power variants of body weight, to allow for curvatures in the relationship. Effects were expressed as marginal effects for a unit increase specific to reference points across the BMI range, with 95% confidence intervals and p-values.

Model fits were checked using Breusch-Pagan/Cook-Weisberg tests for heteroscedasticity, Ramsey's regression specification error tests, and by inspection of normality of residuals and residuals versus fitted values plots. The statistical package "Stata" was used for data handling and analysis. P values less than 0.05 were considered to indicate significance. The procedures followed were in accordance with the (institutional) committee responsible for human experiments.

4. Results

4.1. HBA score and HB-MaSC in clinically infertile men

Our examinations revealed that normozoospermia was found in 59.75% (95 of 159) of the patients in andrological examinations, but the values of HBA% and HB-MaSC index were lower than the published normal values, which underlines the importance of using this improved method. Based on the results below, HB-MaSC is a sensitive indicator of changes in sperm quality and also reflects the proportion of moving sperm.

4.2. Changes of sperm parameters, HBA score and HB-MaSC after supplement therapy

The mean sperm density was 36.55 ± 1.82 (mean ± SEM – Standard Error of Mean) in the untreated group (N = 136). In the treated group (N = 39), in the first analysis, the mean sperm density (10^6/ml) was 24.95 ± 2.91 (mean ± SEM) before supplement administration, and after the therapy, it increased to 35.68 ± 4.32 (mean ± SEM). The difference was significant (p = 0.00805).

In the treated group, in the first analysis, the average HBA rate (%) was 38.71 ± 3.12 (mean ± SEM) and after the supplement therapy, it increased to 49.95 ± 2.99 (mean ± SEM). A statistically significant increase was detected in HBA rate ($p = 0.000004$).

The calculated HB-MaSC value (10^6/ml) was 5.38 ± 0.86 (mean ± SEM) in the first examination, and it was 11.47 ± 1.93 after treatment. A statistically significant increase was observed in this case as well ($p = 0.000015$).

Treated patients were also followed up by telephone 1.5– 2 years after therapy. The partners of 11 treated patients (28.21%) gave birth to healthy babies without assisted reproduction methods. An additional seven partners (17.95%) gave birth with the help of assisted reproductive techniques (intrauterine insemination or intracytoplasmic sperm injection). Consequently, as a result of supplement treatment, 46.15% of the couples previously classified as infertile managed to have babies.

4.3. Correlation between hyaluronan bound matured sperm count (HB-MaSC) and BMI

In the second study, age- and weight-adjusted, regression-fitted values of sperm count were found to be highest at the lower end of the normal BMI range. The sperm count values decreased significantly with the increase of BMI up to 25 kg/m^2, but above this BMI, no remarkable changes were identified, as sperm counts varied to a non-significant extent around a consistently low level of 30 million/ml. This indicates that BMI in the overweight to obese range is associated with a lower sperm count, compared to subjects close to the middle of the normal BMI range. The correlation is best established for body weights up to around 90 kg, but might not be true in case of heavier subjects, who were generally outside the normal BMI range in our sample.

Similar tendencies were observed in the progressively motile sperm rate (WHO-PR), which decreased significantly with the increase of BMI across the normal to overweight range before levelling out in obese subjects. Adjusted for age and BMI, greater body weight, in the 70-90 kg range, was observed to have an elevating eff ect on WHO-PR, with an estimated 17.7 percentage points ($p < 0.0001$) in 80-kg vs. 70-kg, and 11.3 percentage points ($p = 0.0003$) in 90-kg vs. 80-kg subjects of the same age and BMI. No outcome other than WHO-PR was observed to be significantly associated with body weight.

The age- and body weight-adjusted relationship between BMI + HB-MaSC has similar features as BMI + sperm count change linear regression analyses. Values of HB-MaSC were found to

be higher at lower BMI values, decreasing steadily to a BMI of 25 kg/m^2. This decrease is even steeper than that observed for sperm count, which suggests that the BMI + HB-MaSC relationship is more sensitive in illustrating the effect of BMI. Above 25 kg/m^2 BMI, no further decrease in HB-MaSC index was seen, it remained at a low level, which proved to be characteristic for the overweight to obese BMI-range. Body weight seems to be not as closely related to changes in this index as BMI. Although, in the 70-100 kg range, greater body weight showed some tendency to have an elevating effect on HB-MaSC, this was found to be statistically insignificant. The relationship between BMI and HB-MaSC is best established for body weights up to around 100 kg.

Model fit was found to be adequate in all cases, with acceptable minor deviations from normality of residuals in the models for HB-MaSC, sperm count, and WHO-PR; no evidence of poor fit was found for any outcome with other model checking methods.

5. Discussion

It has been shown earlier that simple spermatological tests based on morphology are not sufficient to meet the new requirements of assisted reproductive techniques. Consequently, the value of conventional spermatological examinations in assessing fertility status has been increasingly questioned recently. The hyaluronic binding assay (HBA®) is one of the newly introduced tests that allow functional testing of sperm. The theoretical basis of the HBA® test is that only mature sperm with low frequency of aneuploidy and DNA fragmentation express hyaluronic acid binding receptors (HBR), and only these cells can bind to the hyaluronic acid-containing (HA, also known as hyaluronan) polysaccharide matrix of cumulus cells. Therefore, only mature sperm are able to bind to the hyaluronic acid-treated surface in vitro in HBA assays – as in vivo to the zona pellucida – which makes it possible to distinguish between mature and immature cells. Sperm maturity, low aneuploidy and DNA fragmentation rates, increased chromatin integrity, normal head morphology, and consequently better fertility potential all correlate with hyaluronic acid binding capacity. HA-based sperm selection increases the rate of implantation and reduces the rate of early miscarriages following ICSI, especially in the case of decreased HA-binding capacity of sperm. The hyaluronic binding assay is also useful in oligozoospermia as it helps in the selection of subsequent IVF techniques.

Based on our results, we are convinced that HBA analysis and the index derived from it (HB-MaSC) is an objective, standardisable test that provides a better approach to fertility potential, as it also reflects the proportion of motile sperm. We believe that this improved parameter is

the one that provides the true number of functioning, matured spermatozoa in one mL semen sample. HBA analysis can also be an important diagnostic tool in the case of infertility with normozoospermia. This test allows abnormal samples previously mistaken for normal by the morphological assay to be identified and also makes the efficacy of the therapy much more measurable. In addition, the study helps professionals working in the field of infertility to choose between insemination and ICSI in order to have the best chance of achieving successful reproduction. The authors believe that the use of the HB-MaSC index can describe male fertility more accurately than the conventional parameters used earlier, and it is a potentially useful tool in the hands of the andrologist in everyday andrological practice and prior to assisted reproductive techniques. Our same study also showed that complex dietary supplements containing taurine, magnesium, carnitine, arginine, vitamin C, vitamin E, coenzyme Q10, zinc, beta-carotene, vitamin B6, folic acid, selenium, vitamin B12 and vitamin D3 can be beneficial in the treatment of male infertility. In line with the majority of relevant literature, we found that sperm count and the ability of successful fertilization showed significant improvement due to the complex supplement therapy.

The aim of our second study was to investigate and compare the effect of BMI on conventional sperm parameters (sperm concentration and progressive motility) and hyaluronan binding-based fertility. We examined and compared the effect of BMI on the previously mentioned conventional sperm parameters and hyaluronan binding-based fertilization by evaluating data of 72 infertile men using multiple linear regression analyses adjusted for body weight and age. Based on our results, increased BMI (25 kg/m^2) has a negative effect on sperm count, but body weight itself does not. In the case of progressive motility, a negative effect of increased BMI on WHO-PR was also observed. On the other hand, higher body weights appear to have a less pronounced decreasing effect on WHO-PR – if we compare the curves in the 70-100 kg range – however, at even higher body weights (above 100 kg), the negative effects intensify (although not significantly). Although the same tendency can be seen in the correlations between sperm count and body weight, and HB-MaSC and body weight, the significantly attenuating effect of increasing body weight on WHO-PR decrease was found to be an isolated case: no significant correlation with body weight was observed for results other than WHO-PR. We found that the negative effect of increasing BMI on sperm quality reaches a plateau above 28–30 kg / m2, as sperm concentration, motility, and hyaluronan binding do not decrease further above this BMI. In our experiment, progressive motility was observed to be correlated with BMI, however, this relationship is controversial in the scientific literature, as it was mentioned in the case of BMI and sperm count.

Thus, several new tests for the functional examination of sperm had to be included in research, because conventional spermatological examinations have been shown to be insufficient to assess male fertility. The effect of obesity on male fertility can be better explained by examining the association between BMI and sperm functional variables than with conventional sperm parameters.

The hyaluronan binding assay is a relatively new functional test for assessing the fertility potential of sperm. However, limited data are available on the relationship between overweight and sperm hyaluronan binding capacity. Body mass index can be a preferable candidate for comparison with HBA results, in contrast to body weight, for which the former relationship proved to be not well established in our experiment. It is even better if we compare BMI to another index related to HA binding results, the HB-MaSC, formerly introduced by the authors as a combined interpretation of conventional and functional sperm parameters, which can significantly improve clinical use. This newly described fertility index takes into account sperm density, progressive motility, and HA-binding ability, as a sufficient number of motile spermatozoa is a prerequisite for HA-binding. Using this parameter in the present study, we found that the effect of increased BMI on sperm quality after disregarding body weight is even more pronounced when HB-MaSC is considered.

To the best of our knowledge, the present study provides, for the first time, an analysis of the effect of obesity on the hyaluronan binding capacity of sperm in non-smoking, infertile men. Our results suggest that the effect of body weight changes on hyaluronan binding is less than that exerted by BMI, i.e. an increase in BMI alone (without tobacco use) correlates better with infertility than body weight increase. Even more preferably, our calculation method takes sperm function into consideration, as it uses sperm density, progressive motility and HA-binding capacity values at the same time, thus it provides additional information. HB-MaSC reflects a more explicit relationship between obesity and infertility than body weight or conventional spermatological indicators. Thus, despite the contradictions in the scientific literature regarding the relationship between sperm count and motility, we recommend the use of the HB-MaSC index, which helps to assess fertility status by combining conventional and functional sperm parameters.

6. Summary

Conventional spermatological examinations are cost-effective and quick to perform, but do not provide substantial information for many of today's procedures, while functional tests examine fertility primarily from the perspective of in vitro fertilization. As sperm density is required for treatment strategies and motility affects HA binding capacity, we assume that in everyday andrological practice, the HBA score alone – without knowledge of sperm density and the proportion of progressively moving spermatozoa – does not provide sufficient information to assess male fertility. We found it practically useful to organize the data from the parameters already in use (sperm density, motility, morphology) and the results of the HBA® test into one index. We call this index HB-MaSC (Hyaluronan Bound Matured Sperm Count), which stands for the product of the number of spermatozoa in 1 mL semen, the percentage of progressively motile sperm and the percentile of moving spermatozoa binding to hyaluronic acid.

Based on our results, we are convinced that HBA analysis and the index derived from it (HB-MaSC) is an objective, standardisable test that provides a better approach to fertility potential, as it also reflects the proportion of motile sperm. We believe that this improved parameter is the one that provides the true number of functioning, matured spermatozoa in one mL semen sample, therefore, we recommend the introduction of this index in everyday andrological practice.

As this new index also shows a good correlation with sperm-damaging factors, we recommend the use of this index in andrological research as well.

Complex dietary supplements containing taurine, magnesium, carnitine, arginine, vitamin C, vitamin E, coenzyme Q10, zinc, beta-carotene, vitamin B6, folic acid, selenium, vitamin B12 and vitamin D3 may be beneficial in the treatment of male infertility. In line with the majority of relevant literature, we found that sperm count and the ability of successful fertilization showed significant improvement due to the complex supplement therapy, therefore – where appropriate – we recommend their use in andrological practice.

7. Keywords

sperm quality, BMI, hyaluronan binding assay (HBA®), hyaluronan bound matured sperm count (HB-MaSC)

hyaluronan binding assay, intracytoplasmic sperm injection, male infertility, Picked for Intracytoplasmic Sperm Injection, supplement therapy

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List of publications related to the dissertation

1. **Szűcs, M.**, Osváth, P., Jakab, A., Varga, D., Varga, B., Juhász, B.: Hyaluronan bound mature sperm count (HB-MaSC) is a more informative indicator of fertility than conventional sperm parameters: correlations with Body Mass Index (BMI).
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3. Riesz, P., Rusz, A., **Szűcs, M.**, Majoros, A., Nyirády, P., Keszthelyi, A., Szűcs, M., Mavrogenis, S., Filkor, G., Pánovics, J., Romics, I.: A merevedési zavar megelőzése és kezelésének lehetőségei radikális prostatectomia után.
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