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5. spominski sestanek akad. prof. dr. Lidije Andolšek-Jeras

Premagovanje moške neplodnosti: napredek in novi izzivi

Overcoming Male Infertility: Progress and New Challenges

Zbornik

Ljubljana, oktober 2009

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Zborniku na pot

Letošnji že peti spominski sestanek akademikinje prof. dr. Lidije Andolšek Jeras zaznamujeta dva dogodka. Najprej spomin na našo spoštovano učiteljico, ki se je rodila pred natanko 80 leti in počaščeni smo, da lahko ta dogodek združimo tudi s 1. mednarodnim podiplomskim študijem – Reprodukcija moškega (1st International Master on Male Reproduction).

V sklopu podiplomskega študija smo današnji dan posvetili andrologiji, medicinski veji, ki obravnava reproduktivne funkcije moškega tako v fizioloških kot v bolezenskih pogojih.

Del podiplomskega tečaja, ki ga gosti Klinični oddelek za reprodukcijo Ginekološke klinike Univerzitetnega kliničnega centra v Ljubljani, se je pričel 12. oktobra in se bo končal 30. oktobra 2009.

Pobudo za mednarodni podiplomski študij je dal prof. Carlo Foresta z Univerze v Padovi, k sodelovanju pa so pristopili tudi učitelji Univerz iz Heidelberga (Strowitzki in sod.), Trsta (G. Ricci in sod.), Innsbrucka (Wildt in sod.) in Ljubljane (prof. Borut Peterlin, prof. Gregor Majdič, doc. Irma Virant Klun in doc. Branko Zorn). Tudi študenti tega podiplomskega tečaja so udeleženci iz različnih držav.

V zborniku smo zbrali prispevke priznanih evropskih in slovenskih strokovnjakov s področja andrologije. Namen je bil, da celostno predstavimo obravnavanje neplodnega para s poudarkom na obravnavanju moške neplodnosti, hkrati pa smo želeli predstaviti tudi najnovejše izsledke raziskav, ki posegajo na manj raziskana področja in počasi gradijo mozaik k boljšemu razumevanju andrologije.

Vsem sodelujočim se v imenu Znanstvenega in Organizacijskega odbora najlepše zahvaljujeva, saj bodo tako teoretični kot praktični napotki v pomoč širšemu krogu zdravnikov in biologov, ki se ukvarjajo s humano reprodukcijo.

Doc. dr. Branko Zorn, dr. med.

Predsednik strokovnega odbora

Prof. dr. Eda Bokal Vrtačnik, dr. med.

Predsednica organizacijskega odbora

Foreword

This year annual symposium in the memory of academician prof. dr. Lidija Andolšek Jeras is important for two important reasons. Firstly, this year is 80th anniversary of birth of our esteemed teacher, and we are honoured that we can mark this anniversary with the realization of 1. International postgraduate school about male reproductive medicine – 1st International Master on Male Reproduction.

As a part of this postgraduate course, we have dedicated this day to andrology, field of medicine that deals with male reproductive function in both physiological and diseased circumstances. Part of postgraduate course, hosted by Clinical department for reproduction at Gynaecological clinic at University Clinical Center in Ljubljana, started on 12. October and will conclude on 30. October 2009.

First idea for international master's programme came from prof. Carlo Foresta from University of Padua and after initial idea, teachers from University of Heidelberg (Strowitzki et al.), Trieste (G. Ricci et al.), Innsbruck (Wildt et al.) and Ljubljana (prof. Borut Peterlin, prof. Gregor Majdič, doc. Irma Virant Klun and doc. Branko Zorn) agreed to participate. Students also came from several different countries.

Esteemed European and Slovenian experts in the field of Andrology contributed their works for these proceedings. Our aim was to holistically present how to deal with infertile couple with emphasis on male reproductive problems, together with presenting results from the latest research from different clinical and non-clinical fields that together slowly contribute to our better understanding of whole andrology field.

We would like to thank to all participants on behalf of organizing and scientific committee. Through your willingness to participate in this course and symposium, both theoretical and practical skills will be available to wider group of physicians and biologists, who work in the field of human reproduction.

Assistant Professor Branko Zorn, MD, PhD

President of the Scientific Committee

Associate Professor Eda Bokal Vrtačnik, MD, PhD

President of the Organizing Committee

Vsebina

Genetika moške neplodnosti

Genetics of Male Infertility

Carlo Foresta

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Carlo Foresta je specialist endokrinologije in andrologije ter od leta 2005 redni profesor klinične patologije na Univerzi v Padovi. Od leta 2001 je direktor Centra za zamrzovanje in shranjevanje moških gamet na Padovanski univerzi.

Povzetek

Moška neplodnost je kompleksna bolezen, ki ima v večini primerov genetske osnove. Obstajajo številne raziskave, narejene na modelih mišjih samcev, raziskave o presejanju mutacij in asociacijske raziskave, ki so v zadnjih nekaj letih dokazale visoko prevalenco genetskih vzrokov za slabšo spermatogenezo. 10-15 odstotkov hude moške neplodnosti je posledica genetskih vzrokov zaradi kromosomskih aberacij in mutacij posameznih genov. Naravna selekcija preprečuje prenos teh mutacij, tehnike asistirane reprodukcije pa ta zaščitni mehanizem pogosto zaobidejo. Zato je odkrivanje genetskih dejavnikov postalo del rutinske obravnavnave neplodnega para. Kljub temu pri visokem deležu neplodnih moških vzrok neplodnosti ni prepoznan (nepojasnjena neplodnost), kar odraža naše slabo razumevanje osnovnih mehanizmov, ki uravnavajo spermatogenezo in funkcijo spermijev, zaradi česar težko pravilno diagnosticiramo etiologijo. Ob tem pa molekularni mehanizmi, ki povzročajo okvare spermatogeneze v primerih genetske neplodnosti (na primer mikrodelecije Yq), še vedno niso znani. Te probleme lahko preučujemo le z velikimi asociacijskimi raziskavami in z raziskavami o ekspresiji spermijev in mod, če so spremembe v spermatogenezi jasno določene. Pričakujemo, da bodo te raziskave v bližnji prihodnosti prispevale k pomembnim diagnostičnim in terapevtskim izboljšavam. V tem preglednem članku opisujemo in razpravljamo o genetskih vzrokih moške neplodnosti in genetskih polimorfizmih, ki so verjetno povezani z moško neplodnostjo.

Abstract

Male infertility represents one of the clearest examples of a complex disease with substantial genetic basis. Numerous male mouse models, mutation screening and association studies performed in the last few years definitively demonstrate the high prevalence of genetic causes of spermatogenic impairment. Genetic causes account for 10-15% of severe male infertility, including chromosomal aberrations and single gene mutations. Natural selection prevents the transmission of mutations causing infertility, while this protective mechanism may be overcome by assisted reproduction techniques. Consequently, the identification of genetic factors has become good practice for appropriate management of the infertile couple. However, a large proportion of infertile males are diagnosed as idiopathic, reflecting our poor understanding of the basic mechanisms regulating spermatogenesis and sperm function and hence our inability to properly diagnose the aetiology. Furthermore, the molecular mechanisms underlying spermatogenic damage in cases of genetic infertility (for example Yq microdeletions) are not known. These problems can be addressed only by large scale association studies and testicular or spermatozoal expression studies in well-defined alterations of spermatogenesis. It is conceivable that these studies will provide in the next future important diagnostic and therapeutic implications. In this review we will report and discuss the genetic causes of male infertility known up to date and the genetic polymorphisms possibly associated with male infertility.

Ključne besede: Genetski vzroki, genetski polimorfizmi, kromosomske aberacije, genske mutacije, moška neplodnost, okvara spermatogeneze, mikrodelecije Yq.

Key words: Genetic causes, genetic polymorphisms, chromosome aberrations, gene mutations, male infertility, spermatogenesis impairment, Yq microdeletions.

Introduction

Infertility affects about 15% of couples trying to conceive in Western countries (De Kretser, 1997), and genetic causes may be identified in a large proportion of them. In about 15% of male and 10% of female infertile subjects genetic abnormalities could be present, including chromosome aberrations and single gene mutations. Genetic risks for couples undergoing *in vitro* fertilization and intracytoplasmic sperm injection are related to transmission of constitutional genetic abnormalities, genetic alterations present only in sperm, or *de novo* generated genetic disorders. Therefore, the identification of genetic factors has become good practice for appropriate management of the infertile couple (Foresta *et al*., 2002). Besides known genetic causes of male infertility, recent studies analysed the possible involvement of genetic polymorphisms as risk factors for spermatogenic impairment.

Here we will briefly discuss the most important genetic causes of male infertility (**Table 1**), the role of genetic polymorphisms, and finally, we will present novel studies aimed at identifying genetic defects of human spermatogenesis by means of testicular expression microarray analysis.

Table 1. *Frequency and associated phenotypes of the most common genetic abnormalities associated with male infertility. The prevalence shown is the frequency found in the associated phenotype.*

Genetic abnormality	Phenotype	Prevalence
Chromosomal aberrations	From azoospermia to normospermia	2-10%
Klinefelter syndrome	Azoospermia-severe oligospermia	5-10% azoospermia 2-5% severe oligospermia
Other sex chromosome alterations	From azoospermia to normospermia	0.1-0.2%
Robertsonian translocations	Azoospermia-severe oligospermia	0.5-1.0%
Reciprocal translocations	Azoospermia-severe oligospermia	0.5-1.0%
Y chromosome deletions	Azoospermia-severe oligospermia	5-10%
AZFa	Azoospermia-SCOS	0.5-1.0%
AZFb	Azoospermia-spermatogenic arrest	0.5-1.0%
AZFc	Azoospermia-severe oligospermia	3-7%
AZFb-c	SCOS/Spermatogenic arrest	0.5-1.0%
Partial AZFc deletions	From azoospermia to normospermia	3-5%
Gene mutations		
CFTR	Obstructive azoospermia	60-70% (5% in infertile men)
AR	Azoospermia-oligospermia	2-3%
INSL3-LGR8	Cryptorchidism	4-5%

SCOS: Sertoli cell only syndrome.

Genetic causes of male infertility

Chromosome abnormalities

The prevalence of chromosome abnormalities is higher in infertile men, this figure being inversely related to the sperm count. Based on the largest published series it could be estimated that the overall incidence of a chromosomal factor in infertile males ranges between 2 and 8%, with a mean value of 5%. This value is increasing to about 15% in azoospermic males, being largely contributed by patients with 47,XXY Klinefelter's aneuploidy. Sex chromosomes abnormalities are predominating, but a wide range of structural autosomal anomalies are also found. The most common type of karyotype abnormality detected in infertile subjects is represented by Klinefelter syndrome (KS) (Foresta *et al.*, 2002). In a series of 750 consecutive severe oligozoospermic men (sperm count<5 million/ml) collected in our Centre from 1996 (Foresta *et al.*, 2005), chromosome aberrations were present in 42 subjects (5.6%) of which 29 (69.0%) were of the Klinefelter's type.

KS is the most frequent sex chromosome aneuploidy in human males, occurring in approximately 0.1-0.2% of newborn males. The prevalence of KS among infertile men is very high, up to 5% in severe oligozoospermia and 10% in azoospermia. KS is a form of primary testicular failure with testicular hypotrophy and elevated gonadotropin plasma levels, and it represents the most common form of male hypogonadism.

It has been always assumed that almost 100% of non-mosaic 47,XXY males are azoospermic. However, in our series, 72 of the 94 (76.6%) non-mosaic KS had complete azoospermia, whereas the remaining had sperm in the ejaculate. Although clear data are not available, it is also possible that a fraction of azoospermic KS men might actually have residual spermatogenesis in some seminiferous tubules (Foresta et al., 1998). Mosaic 47,XXY/46,XY patients produce spermatozoa in variable numbers. Although the exact percentage of men with sperm in the ejaculate is not known, in our series 20 of the 27 (74.4%) were azoospermic.

Before the introduction of ICSI, the fertility outlook for the vast majority of KS patients was hopeless. To date, 54 normal children have been born from 122 men with KS by ICSI with testicular (48 children, 118 patients) or ejaculated spermatozoa (6 children from 4 patients) (reviewed in Ferlin *et al.*, 2005). Although a great majority of children born to fathers with KS are chromosomally normal, the risk of producing offspring with chromosome aneuploidies is significant, particularly the risk of fathering a 47,XXY or 47,XXX child (Reubinoff *et al.*, 1998; Ron.El *et al.*, 2000). In fact, the incidence of aneuploid spermatozoa (particularly of disomies) is increased in KS (Ferlin *et al.*, 2005). Aneuploid spermatozoa are probably the result of meiosis of few 47,XXY spermatocytes and of meiotic abnormalities occurring in normal 46,XY germ cells present in a compromised testicular environment (Ferlin *et al.*, 2005).

Other sex chromosomes aneuploidies detected with higher prevalence in infertile men are represented by 47,XYY, 46,XX and Y chromosome aberrations (inversions, Yq deletions,

etc). Furthermore, translocations involving the sex chromosomes (X-autosomal and Y-autosomal translocations) are also frequent (Mau-Holzmann *et al.*, 2005).

Robertsonian translocations are the most frequent structural chromosomal abnormalities in humans and can affect fertility with various degrees of sperm alterations in men. Robertsonian translocations occur when two acrocentric chromosomes (Vogt *et al.*, 1996; Kuroda-Kawaguchi *et al.*, 2001; Jiang *et al.*, 1999; Stuppia *et al.*, 2001; Stouffs *et al.*, 2005) fuse together. The resulting single abnormal chromosome, generally dicentric, contains most of the long arms of the original two and subsequent loss of their short arms. The incidence of Robertsonian translocations is ~1 in 1000 newborns (Therman and Susman, 1993). The most common combinations are between chromosomes 13 and 14 and between chromosomes 14 and 21. Carriers of the Robertsonian translocation generally have normal phenotypes. However, the translocation can affect fertility and/or pregnancy outcome due to possibly impaired gametogenesis and/or production of gametes with an unbalanced combination of the parental rearrangement. Fertility problems in Robertsonian translocation male carriers are due to various degrees of spermatogenic defects directly related to the disturbance of the meiotic process. In populations of infertile males 0.8% were carriers of a Robertsonian translocation (De Braekeleer and Dao, 1991), this is up to nine times higher than in the general population.

Reciprocal translocations are found with a frequency of 0.9/1000 newborns. A translocation consists of a mutual exchange of chromosomal segments between two chromosomes. In general, there is no apparent alteration to the carrier's phenotype. However, in couples experiencing repeated pregnancy losses, the incidence of chromosomal translocations is higher than the incidence present in newborn series (De Braekeleer and Dao, 1991). On the other hand, there is also evidence which indicates that the presence of translocations alters the spermatogenic process. Summarizing the findings from different series of studies on infertile, oligozoospermic and azoospermic males, the incidence of reciprocal translocation carriers is seven times more elevated than in newborn series.

Y chromosome microdeletions

Microdeletions in the Y chromosome long arm (Yq) represent the most frequent molecular genetic cause of severe infertility, observed with a prevalence of 10-15% in non-obstructive azoospermia and 5-10% of severe oligozoospermia (Foresta *et al.*, 2001). Most of the deletions are found in men with a sperm count below 2 million/ml (Ferlin *et al.*, 2007). Three regions, referred to as “azoospermia factors” (AZFa, b and c from proximal to distal) have been defined as spermatogenesis loci (Vogt *et al.*, 1996). The genetic pathways and mechanisms of spermatogenic impairment in men with Yq microdeletions are unknown. The function of AZF genes in spermatogenesis is also not clear, and the molecular mechanisms altered in cases of AZF deletions are completely unknown. The majority of Y microdeletions produce a simultaneous loss of several genes mapped within AZFb and AZFc loci (Ferlin *et al.*, 2007; Kuroda-Kawaguchi *et al.*, 2001; Stuppia *et al.*, 2001; Repping *et al.*, 2002). AZFa deletions are less frequent and involve only two genes, USP9Y and DBY. Most of the AZF microdeletions are generated by intrachromosomal homologous recombi-

nation between repeated sequence blocks organised into palindromic structures showing a nearly identical sequence (Kuroda-Kawaguchi *et al.*, 2001; Repping *et al.*, 2002). The complete AZFc deletion, b2/b4 deletion, removes eight gene families including all members of the DAZ gene family, that represent the strongest candidate responsible for the AZFc phenotype (Reijo *et al.*, 1995; Foresta *et al.*, Kuroda-Kawaguchi *et al.*, 2001; Repping *et al.*, Oates *et al.*, 2002; Simoni *et al.*, 2004). Deletions in the AZFa region usually lead to Sertoli cell-only syndrome, complete deletions of AZFb or AZFb+c lead to azoospermia associated with Sertoli cell-only syndrome or pre-meiotic spermatogenic arrest (VOGT *et al.*, 1996; Foresta *et al.*, 2001; Ferlin *et al.*, 2007;). The most frequent AZFc deletion leads to azoospermia or severe oligozoospermia, associated with different spermatogenic phenotypes in the testis. In general, 60-70% of these men have sperm in the ejaculate or in the testis (Ferlin *et al.*, 2007).

Most men with Yq microdeletions require ICSI to overcome their infertility. Since all spermatozoa from Y-deleted men harbour the same microdeletions, ICSI allows the transmission of such microdeletions. Male offspring of men with Yq microdeletions will therefore also carry the deletion and will have spermatogenic impairment in adulthood. However, a recent acquisition is that men with AZFc also produce a higher percentage of sperm with aneuploidies. In fact, we recently reported that patients with AZFc deletions had a significant reduction in the percentage of normal Y-bearing spermatozoa with respect to normozoospermic control men, a concomitant increase in nullisomic sperm and a significant increase of XY-disomic sperm (Foresta et al., 2005; Ferlin et al., 2007). Therefore, AZF microdeletions can be considered as "pre-mutations" for a subsequent complete loss of the Y chromosome in the AZF deleted patients' sperm, increasing the risk of embryonic X0 cells (Vogt, 2004).

Although no genital abnormalities or other somatic defects in the ICSI-AZFc offspring have been reported, genetic counselling should take into account the observations of sperm sex chromosome aneuploidies in these men, and a possible increased risk of generating 45,X (Turner syndrome) or 47,XXY embryos (Klinefelter syndrome). It has to be noted, however, that these risks are more theoretical because in the 31 children already born from men with AZF deletion (Jiang *et al.*, Page *et al.*, 1999; Cram *et al.*, 2000; Komori *et al.*, 2002; Stouffs *et al.*, 2005) no consequences other than transmission of the Yq deletion have been reported. Nevertheless, clear information regarding implantation rate and incidence of spontaneous abortion for the partners of men with Yq microdeletions is not available.

Gene mutations

Several hundreds of genes are necessary for normal sexual development, testis determination, testis descent, and spermatogenesis. However, only few of them have routine clinical importance. These include the CFTR gene, whose mutations cause cystic fibrosis and absence of vas deferens, the androgen receptor gene, whose mutations cause the androgen insensitivity syndrome and spermatogenic damage, and the INSL3-LGR8 genes, whose mutations have been associated with abnormalities in the testis descent (cryptorchidism).

There is general agreement that 60%-70% of patients with congenital bilateral absence of the vas deferens (CBAVD) have mutations in the CFTR gene, with no other clinical symptoms of cystic fibrosis. In our series of unselected severely oligozoospermic men (Foresta *et al.*, 2005) we found a prevalence of 1.2% (9/750) of CFTR gene mutations. A recent survey in Italy found CFTR mutations in 37.5% of CBAVD individuals and 6.6% in males with nonobstructive azoospermia (Stuppia *et al.*, 2005). Furthermore, the 5T allele was found with high prevalence both in males with nonobstructive-azoospermia (9.9%) and in those with CBAVD (100%). All together, 11.6% of subjects entering assisted reproductive techniques had either a CFTR mutation or the 5T allele. Subjects with CFTR mutations are good candidates for ICSI, using sperm retrieved from the ejaculate, testis, or epididymis. Spermatogenesis in these patients is assumed to be normal, and the aneuploidy rate is not increased in the sperm of affected patients. However, because of the risk of cystic fibrosis in the offspring of couples in which the female partner is heterozygous for a CFTR mutation, screening for CFTR mutations should be considered before assisted reproduction techniques (Foresta *et al.*, 2002).

Mutations in the androgen receptor (AR) gene on the X chromosome cause a variety of defects known collectively as androgen insensitivity syndrome (AIS). Patients with mild AIS (MAIS) have male infertility as their primary or even sole symptom. In a recent screening of 1517 azoo-oligozoospermic individuals we found 26 patients carrying AR mutations (20 different mutations) (1.7%), and none in the control group (Ferlin *et al.*, 2006a). Importantly, of the 26 men with AR gene mutations, two had cryptorchidism, one cryptorchidism and hypospadias, one gynecomastia, whereas 22 did not show signs of androgen insensitivity other than spermatogenic impairment. Furthermore, only a minority of infertile males with elevated testosterone and LH (suggestive for androgen insensitivity) had mutations in the AR gene, even thought the higher the ASI (androgen sensitivity index, the product of LH x testosterone), the more likely a mutation in the AR. Therefore, AR gene mutations might play a role as genetic cause of male infertility and are found with a prevalence of about 2% in unselected infertile men, with similar prevalence in azoospermia, severe oligozoospermia and moderate oligozoospermia (Ferlin *et al.*, 2006a). No clear hormonal or clinical data could be used to preselect patients at higher risk of mutations. Thought mild signs of androgen insensitivity may be present in some cases, the largest part of men with AR abnormalities do not differ from the vast majority of infertile males.

Insulin-like factor 3 (INSL3) is a member of the relaxin-like hormone family produced by the Leydig cells. Research on INSL3 in humans has expanded in the last years following the identification, in rodents, of a role for this peptide in the transabdominal phase of testicular descent by acting on gubernaculum (Nef and Parada, Zimmermann, 1999). A further impulse to this research has been given by the description of the relative receptor, LGR8 (leucine-rich-repeat-containing G protein-coupled receptor 8) (Overbeek *et al.*, 2001; Bogatcheva *et al.*, 2003). A role in human cryptorchidism has been suggested since several mutations in INSL3 and LGR8 leading to amino acid substitution were found. A review of the literature found a prevalence of mutations of 4-5% in men with cryptorchidism or ex-cryptorchidism (Ferlin and Foresta, 2005). Some of these mutations represent common

polymorphisms found with similar frequency both in patients and controls, whereas seven of them were detected exclusively in men with a history of maldescent (P49S, R73X, P93L, R102C, R102H, N110K in INSL3 and T222P in LGR8). Moreover, we found a significant association of INSL3 gene mutations in men presenting one or more signs of the testicular dysgenesis syndrome (Ferlin *et al.*, 2006b). However, a causative role for some of these mutations is not clearly supported by functional analyses. Therefore, although a role for mutations of INSL3 and LGR8 genes in cryptorchidism is reasonable, additional studies are needed to establish an association between the disruption of INSL3 pathway and a higher risk of infertility or testicular cancer. Finally, the identification of high levels of circulating INSL3 in adult males and the expression of LGR8 in many tissues opens new question about the endocrinological role of this hormone (Foresta *et al.*, 2004; Bay *et al.*, 2005; Ferlin *et al.*, 2006c). Apart from the role in testicular descent and cryptorchidism, INSL3 has therefore possible important yet unidentified endocrine and paracrine actions in adults, whereas the deficiency of this hormone may represent an important sign of functional hypogonadism (Foresta *et al.*, 2004; Ferlin and Foresta, 2005; Ferlin *et al.*, 2006b; Ferlin *et al.*, 2006d).

Conclusions

Male infertility represents one of the clearest examples of a complex disease with substantial genetic basis. Numerous male mouse models, mutation screening and association studies performed in the last few years definitively demonstrate a high prevalence of genetic causes of spermatogenic impairment. However, a large proportion of infertile males are diagnosed as idiopathic, reflecting our poor understanding of the basic mechanisms regulating spermatogenesis and sperm function, and hence our inability to properly diagnose the aetiology. Furthermore, the molecular mechanisms underlying spermatogenic damage in cases of genetic infertility (for example Yq microdeletions) are not known. These problems can be addressed only by large scale association studies and testicular or spermatozoal expression studies in well-defined alterations of spermatogenesis. It is conceivable that these studies will provide in the next future important diagnostic and therapeutic implications.

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Selekcija spermijev za ICSI na podlagi njihove morfologije

Sperm Selection Before ICSI Based On Their Morphology

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Raziskovalno je dejavna na področju matičnih celic v humanih gonadah in gametogeneze in vitro.

Povzetek

Z analizo kakovosti pripravljenega semena, ki ostane po postopku ICSI, smo ugotovili pozitivno korelacijo med deležem spermijev z nenormalno morfologijo glave in deležem zarodkov, ki so se razvojno zaustavili pri podaljšanem gojenju in se niso razvili do razvojne stopnje blastociste ali morule. Glavni namen te raziskave je bil ugotoviti, pri kakšnem deležu moških z nenormalno morfologijo spermijev (teratozoospermijo), vključenih v program ICSI, je pod 6000-kratno povečavo možno najti normalne spermije brez vakuol v glavi. S sistemom IMSI smo pri spermijih po klasifikaciji Cassuto-Barak ocenjevali morfologijo glave (normalna = 2 točki), morfologijo baze (normalna = 1 točka) in odsotnost vakuol v glavi (ena majhna vakuola ali brez = 3 točke). Popolnoma normalen spermij (ali z eno majhno vakuolo) smo ocenili s 6 točkami. Spermije smo razdelili v tri razrede: I (6-4 točk), II (3-1 točka) in III (0 točk). Spermiji I. in II. razreda so bili primerni za injiciranje v jajčne celice, spermiji III. razreda pa ne. Optimalne spermije I. razreda smo lahko našli pri 44 % bolnikov, pri ostalih pa smo lahko našli samo spermije II. razreda. Spermije III. razreda smo našli pri vseh bolnikih. Našli smo povprečno 1.8% spermijev I. razreda in 7.5% spermijev II. razreda na bolnika. Morfološko normalne spermije brez vakuol v glavi je torej možno najti pri manj kot polovici moških s teratozoospermijo, vključenih v program ICSI. Preliminarna raziskava selekcije spermijev za postopek ICSI z metodo IMSI pri manjšem številu bolnikov s skrajno obliko teratozoospermije (≤ 5 % morfološko normalnih spermijev) je pokazala, da s selekcijo spermijev z IMSI kljub kompleksni predpripravi spermijev ne

poslabšamo izida postopka ICSI. Raziskavo nadaljujemo na večjem številu bolnikov s teratozoospermijo.

Abstract

After the analysis of the prepared semen quality after ICSI there was a positive correlation between the proportion of spermatozoa with sperm head morphological abnormalities and the proportion of arrested embryos which did not develop to the blastocyst or morula stage after the extended in vitro culture. The main purpose of this study was to evaluate the proportion of men with abnormal sperm morphology (teratozoospermia) included in the ICSI program who had spermatozoa without head vacuoles, as revealed at 6000 times magnification. The morphology of the sperm head (normal = 2 points), head basis (normal = 1 point), and the absence of vacuoles in the sperm head (1 small vacuole or without it = 3 points) was evaluated by the system IMSI according the Cassuto-Barak classification. Each spermatozoon with a completely normal morphology was evaluated by 6 points. Spermatozoa were divided into 3 different classes: I (6-4 points), II (3-1 points), and III (0 point). Spermatozoa of the classes I and II were appropriate for injection into the oocytes, whereas spermatozoa of class III were not. Optimal spermatozoa of class I were found in 44% of patients with teratozoospermia; in others only spermatozoa of class II were found. Spermatozoa of class III were found in all patients. The average proportions of class I and class II spermatozoa were 1.8% and 7.5% per patient. Morphologically normal spermatozoa were found in less than a half of men with teratozoospermia included in the ICSI program. The preliminary selection of spermatozoa by the IMSI method did not decrease the results of ICSI in men with severe teratozoospermia (≤ 5% of morphologically normal spermatozoa) in spite of the complex preparation of spermatozoa for microinjection. This study is ongoing on a higher number of patients.

Ključne besede: ICSI, IMSI, klasifikacija, morfologija, selekcija, spermij, vakuola.

Key words: ICSI, IMSI, Cassuto-Barak classification, morphology, selection, sperm, vacuole.

Uvod

Metoda neposrednega vnosa spermija v citoplazmo jajčne celice (ICSI) je izjemna, saj omogoča oploditev jajčnih celic s spermiji slabe kakovosti in s tem zdravljenje najtežjih oblik moške neplodnosti. Do otroka lahko pridejo tudi moški, ki zaradi slabe kakovosti semena ne bi naravno nikoli spočeli otroka. Kljub številnim prednostim metode pa se postavlja vprašanje, kaj dejansko pomeni oploditev jajčnih celic s spermiji slabe kakovosti brez naravne selekcije. Metoda ICSI namreč izključuje naravno selekcijo spermijev. Spermij, ki ga injiciramo v jajčno celico, je izbran izmed množice spermijev v pripravljenem semenu. Umetno selekcijo spermija izvedemo pri povečavi 200-400-krat, ki ne omogoča korektne selekcije spermijev glede na morfologijo. Znano je, da je skrajna teratozoospermija povezana z večjo pojavnostjo anevploidij pri spermijih (Faure in sod., 2007). Kljub temu so rezultati različnih raziskav, ki so iskale povezavo med morfologijo spermijev in izidom postopka ICSI, zelo nasprotujoči. Nekateri avtorji niso ugotovili nobene povezave med mor-

fologijo spermijev in izidom postopka ICSI (Mansour in sod., 1995; Nagy in sod., 1995; Oehninger in sod., 1998), drugi pa so ugotovili negativen vpliv skrajne teratozoospermije (Tasdemir in sod., 1997), globozoospermije (Liu in sod., 1995) in megalozoospermije (Kahraman in sod., 1999) na razvoj zarodkov in zanositev po ICSI. V zadnjem času je bilo razvitih več metod za selekcijo spermijev, ki naj bi izboljšale izid postopka ICSI. Ena od teh metod je IMSI (angl. *Intracytoplasmic morphologically selected sperm injection*), ki temelji na selekciji spermijev predvsem na osnovi vakuol v njihovih glavah, ki so povezane s porušeno integriteto DNA v jedru in slabšo funkcionalnostjo (Bartoov in sod., 1994; Bartoov in sod., 2003; Berkovitz in sod., 2005; Vanderzwalmen in sod., Cassuto in sod., 2008). Kljub publikacijam pa natančnega mesta IMSI v klinični praksi še ni možno opredeliti. Namen te raziskave je bilo ugotoviti mesto IMSI v programu zdravljenja neplodnosti.

Material in metode

Raziskava je bila izvedena na Ginekološki kliniki, Univerzitetni klinični center Ljubljana, v časovnem obdobju junij 2008 do junij 2009. Namen raziskave je bil odgovoriti na tri pomembna vprašanja: ali morfologija spermijev v vzorcu pripravljenega semena vpliva na izid postopka ICSI, pri kolikšnem deležu bolnikov s teratozoospermijo v programu ICSI je možno pod 6000-kratno povečavo s sistemom IMSI dobiti morfološko normalne spermije I. in II. razreda, in kakšen je izid postopka IMSI pri bolnikih s skrajno teratozoospermijo (≤ 5 % morfološko normalnih spermijev). Klasična morfologija spermijev (glava, vrat, srednji del, rep) je bila ocenjevana po barvanju razmaza semena po Papanicolaou in vrednotena po kriterijih Svetovne zdravstvene organizacije. S sistemom IMSI (dicNomarski imerzijski objektiv, digitalna kamera) smo na invertnem mikroskopu (Nikon, Japonska) pod 6000-kratno povečavo selekcionirali spermije po Cassuto-Barakovi klasifikaciji (Cassuto in sod., 2008). Ocenjevali smo morfologijo glave (normalna = 2 točki), morfologijo baze (normalna = 1 točka) in odsotnost vakuol v glavi (ena majhna vakuola ali brez = 3 točke). Popolnoma normalen spermij (ali z eno majhno vakuolo) smo ocenili s 6 točkami. Spermije smo razdelili v tri razrede: I (6-4 točk), II (3-1 točka) in III (0 točk). Spermiji I. In II. Razreda so bili primerni za injiciranje v jajčne celice, spermiji III. razreda pa ne. Pripravljene spermije smo za selekcijo upočasnili v gojišču SpermSlow (MediCult, Danska) in jih opazovali v injekcijski pipeti. Postopek ICSI smo izvajali po že ustaljeni metodi (Virant-Klun in sod., 2002). Zarodki so bili gojeni v gojiščih Cook (Avstralija) do razvojne stopnje blastociste. Eno ali največ 2 blastocisti smo prenesli v maternico, nadštevilne blastociste pa shranili z zamrzovanjem.

Rezultati

Vpliv nepravilnosti morfologije glav spermijev na razvoj zarodkov v postopku zunajtelesne oploditve

Pri 52 bolnikih smo naredili razmaz vzorca pripravljenega semena, ki je ostalo po postopku ICSI in ga barvali po Papanicolaou za klasično oceno morfologije spermijev. Po primerjavi z izidom postopka ICSI smo ugotovili statistično značilno pozitivno korelacijo med nepravilnostmi glave spermijev in deležem zarodkov, ki so se med podaljšanim gojenjem razvojno

zaustavili in se niso razvili do blastociste ali morule oziroma niso bili primerni za prenos v maternico ali shranjevanje z zamrzovanjem. (**Tabela 1**). To smo potrdili tudi z regresijsko analizo, kjer smo upoštevali starost ženske, indikacije neplodnosti pri ženski in število pridobljenih jajčnih celic. Preostale morfološke nepravilnosti vratu, srednjega dela in repa spermijev niso vplivale na izid postopka ICSI.

***Tabela 1**. Povezava med klasično morfologijo spermijev in izidom postopka ICSI.*

	Število zaustavljenih zarodkov	Število zavrženih zarodkov
Normalni spermiji (sp.) (%)	0.926	0.975
Nenormalni sp. (%)	0.008*	0.011*
Sp. z nepravilnostmi glave (%)	0.014*	0.022*
Sp. z nepravilnostmi vratu (%)	0.576	0.324
Sp. z nepravilnostmi srednjega dela (%)	0.421	0.592
Sp. z nepravilnostmi repa (%)	0.281	0.261

*statistično značilne razlike, ugotovljene s Spearmanovim korelacijskim koeficientom ($P<0.05$).

Vakuole v glavah spermijev, ocenjene z metodo IMSI pri bolnikih s teratozoospermijo v postopku ICSI

Pripravljeno seme, ki je ostalo po postopku ICSI, smo pregledali s sistemom IMSI pri 20 bolnikih s teratozoospermijo (samo ali v kombinaciji z drugimi nepravilnostmi semena). Spermije I. razreda (**Slika 1A**) smo lahko našli pri 9 bolnikih (45 %), pri ostalih (55 %) pa smo lahko našli samo spermije II. razreda (**Slika 1B**) in (ali) III. razreda (**Slike 1C, D, E**). Spermije III. razreda smo našli pri vseh bolnikih. Našli smo povprečno 1,8 spermija I. razreda in 7,5 spermijev II. razreda na moškega. Optimalne spermije I. razreda smo torej našli pri manj kot polovici bolnikov s teratozoospermijo. Slike se nahajajo v dodatku.

Selekcija spermijev za ICSI z metodo IMSI pri bolnikih s skrajno teratozoospermijo

Opravili smo selekcijo spermijev z metodo IMSI in izvedli ICSI s selekcioniranimi spermiji v prvi skupini bolnikov – pri 6 bolnikih s skrajno obliko teratozoospermije oziroma z ≤ 5 % morfološko normalnih spermijev v semenskem izlivu. Rezultate smo primerjali s skupino bolnikov s skrajno teratozoospermijo, pri katerih smo izvedli običajno metodo ICSI v enakem časovnem obdobju. Ugotovili smo, da injiciranje spemijev, selekcioniranih z metodo IMSI, kljub kompleksni predobravnavi spermija ne poslabša izida postopka ICSI (**Tabela 2**). Ovrednotenje kliničnega doprinosa selekcije spermijev z metodo IMSI pri skrajni teratozoospermiji bo mogoče v prihodnosti na večjem številu bolnikov ob poznavanju poteka nosečnosti in zdravja otrok, rojenih po tej metodi.

***Tabela 2**. Primerjava rezultatov ICSI in IMSI pri bolnikih s skrajno teratozoospermijo (≤ 5 % morfološko normalnih spermijev).*

	ICSI	IMSI
Število postopkov	72	6
Starost ženske (leta)	33,9 ± 4,0	34,1 ± 4,5
Število zarodkov	354	17
Število zarodkov, ki so se razvojno zaustavili (%)	205 (58,0)	9 (52,9)
Število nosečnosti	23	2
Stopnja zanositve na aspiracijo jajčnih celic	32 %	33 %
Stopnja zanositve na prenos blastociste (%)	49 % (23 / 47)	100 % (2 / 2)

Razpravljanje

Selekcija spermijev s sistemom IMSI je zelo zahtevna in zamudna, predvsem pri bolnikih s skrajno teratozoospermijo, saj je normalne spermije težko najti. Ocenili smo, da je pri manj kot polovici teh bolnikov možno dobiti spermije I. in II. razreda, ki so primerni za ICSI. Pregled spermijev s sistemom IMSI pod 6000-kratno povečavo ima diagnostičen pomen. Skupino z velikim tveganjem (npr. za spontani splav ali potencialno razvojno nepravilnost pri otroku) predstavljajo bolniki, pri katerih lahko s postopkom ICSI injiciramo samo spermije III. razreda, ker spermijev boljše kakovosti ni. Morda bi bila pri teh bolnikih priporočljiva predimplantacijska genetska diagnostika zarodkov v postopku ICSI. V prihodnosti bo potrebno razmišljati tudi o drugih skupinah bolnikov, primernih za diagnostiko vakuol v glavah spermijev kot so na primer moški z normalno kakovostjo semena, ki dosegajo zelo slabo oploditev jajčnih celic oziroma moški z idiopatsko neplodnostjo.

Ugotovitev, da nenormalna morfologija glav spermijev negativno vpliva na razvoj zarodkov po postopku ICSI, nas ni presenetila. S to ugotovitvijo smo potrdili ugotovitve številnih predhodnih raziskav, ki so ugotavljale isto (Jones in sod., Shoukir in sod., 1998; Miller in sod., 2001; Rawe in sod., 2002; Tesarik, 2005; Loutradi in sod., 2006). V naši raziskavi je bila ugotovljena pozitivna korelacija med deležem spermijev z nenormalno morfologijo glav v vzorcu semena, pripravljenega za ICSI, in razvojno zaustavljenimi zarodki po ICSI, ki se kljub podaljšanem gojenju niso razvili do razvojne stopnje blastociste in morule in niso bili primerni ne za prenos v maternico, ne za shranjevanje z zamrzovanjem. To je zelo verjetno odražalo genetske nepravilnosti spermijev z nenormalno morfologijo glave.

Selekcija spermijev glede na vakuole v glavah je pomembna in smiselna, saj obstaja povezava vakuol s poškodbami kromatina oziroma DNA spermijev (Berkovitz in sod., 2006; Gopalkrishnan in sod., 2000; Hazout in sod., 2006; Franko in sod., 2008; Vanderzwalmen s sod., 2008). Dokazano je bilo tudi, da imajo spermiji z normalno morfologijo akrosoma, glave, vratu in repa in brez vakuol v glavi boljšo mitohondrijsko funkcijo in manj porušene integritete DNK (npr. fragmentacije DNK) kot spermiji z normalno morfologijo, a vsaj 1 vakuolo v glavi. Analiza s fluorescenčno *in situ* hibridizacijo je pokazala, da pri bolnikih z

oligozoospermijo spermiji z normalno morfologijo in brez vakuol nimajo anevploidij (Garolla s sod., 2008).

Prvi poskusi injiciranja spermijev, selekcioniranih s sistemom IMSI, pri bolnikih s skrajno obliko teratozoospermije, so pokazali, da se kljub kompleksni predpripravi spermija za selekcijo in sam tehnični postopek selekcioniranja ne poslabša izida ICSI. Šele tovrstna selekcija spermijev na večjem številu bolnikov bo omogočila korektno ovrednotenje njenega kliničnega učinka in pomena. Predpostavljamo, da bo izvajanje IMSI predstavljalo varnejši način izvajanja ICSI pri bolnikih z največjim tveganjem kot so na primer moški s skrajno teratozoospermijo. To naj bi se odrazilo v boljšem izidu nosečnosti z manj spontanimi splavi in v zdravju otrok.

Zaključili bi lahko, da je IMSI nedvomno pomembna in perspektivna metoda na področju zdravljenja moške neplodnosti.

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Izbira spermija v IVF laboratoriju

Selection of sperm in the IVF laboratory

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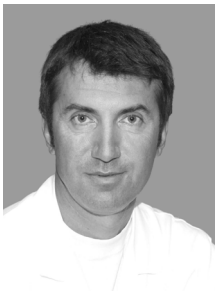

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Povzetek

Pri zdravljenju neplodnosti z zunajtelesno oploditvijo imata laboratorijska priprava semena in izbira semenčic za oploditev jajčnih celic zelo pomembno vlogo. Izbor semenčic vpliva na stopnjo oplojenosti, embrionalni razvoj in uspešnost postopka zdravljenja. Namen prispevka je prikazati številne laboratorijske metode, s katerimi se je iz semenskega izliva poskušalo izolirati najoptimalnejše semenčice. Opisane so prednosti in slabosti "swim-up" metode, ki jo najpogosteje uporabljamo pri klasični oploditvi in vitro. Naštete so tudi številne različice, ki so se sčasoma razvile iz te osnovne metode in so primerne v posebnih primerih, kot so težka oligoastenoterato- in kriptozoospermija. Opisane so tehnike za lažje iskanje in prepoznavanje živih semenčic iz vzorcev semena, kjer je gibljivost najbolj problematična. To so vzorci s popolnoma negibljivimi semenčicami in vzorci tkiva mod. Razvoj optične tehnologije in biotehnologije je izboljšal možnosti izbora morfološko najoptimalnejših semenčic in tistih z nepoškodovano DNK.

Abstract

Laboratory semen preparation and sperm selection have an important role in infertility treatment by in vitro fertilization (IVF) methods. Sperm selection influences the fertilization rate, embryo development and success rates of the treatment. The aim of this paper is to review the numerous laboratory methods, by which the optimal sperm can be isolated from the semen. The advantages and drawbacks of the "swim-up" technique – the most frequently used sperm washing method in classical IVF – are described. Numerous modifications, which have developed from the swim-up method are described as well. They are

suitable for preparation of oligoastheno- or cryptozoospermic semen samples. The techniques for easiest searching and identification of viable sperm in the samples with sperm motility problem are shown too. These are the samples with total sperm immotility and testicular sperm samples. The developments of optical technology and biotechnology have increased the possibilities for the selection of the morphologically best sperm and those with undamaged DNA.

Ključne besede: oploditev in vitro, intracitoplazmatsko injiciranje semenčice, tehnike priprave semenčic, izbira semenčic, oploditvena sposobnost semenčic.

Key words: in vitro fertilization, intracytoplasmic sperm injection, sperm preparation techniques, sperm selection, sperm fertilizing capacity.

Uvod

V postopku oploditve z biomedicinsko pomočjo (OBMP) ima laboratorijska obdelava semena moških svoje mesto. S posebnimi centrifugalnimi tehnikami se iz semena odstrani semenska plazma, celični debris, bakterije in negibljive celice, kot so mrtvi spermiji, levkociti, celice spermatogeneze, epitelne celice ter kristali in drugi želatinasti delci, ki preprečujejo oploditev ali pa zaradi sproščanja prostih kisikovih radikalov poslabšajo pogoje kultiviranja, na katere je jajčna celica še posebej občutljiva. Da bi se iz semena dobilo čim večje število dobro gibljivih spermijev, so v preteklosti, pred 15 leti, ko še ni bilo razvite metode neposrednega injiciranja semenčice v citoplazmo jajčne celice (ICSI), razvili celo vrsto različnih laboratorijskih separacijskih metod, temelječih večinoma na centrifugiranju semena skozi stolpec gojišča. Njihova učinkovitost se je merila predvsem po številu in čistosti dobljenih gibljivih spermijev, vendar pa v primerih težje oligo- ali astenozoospermije pogosto tudi s temi tehnikami ni bilo možno doseči oploditve jajčnih celic.

Ob koncu 80 let prejšnjega stoletja so mnogi poskušali izboljšati kakovost semena oz. gibljivost semenčic z uporabo različnih stimulatorjev gibanja, kot so kafein, kalikrein, 2-deoksiadenozin in pentoksifilin. Predvsem slednji se je v OBMP precej razširil.

Metoda ICSI je poenostavila tehnike priprave semena, saj je bilo potrebno najti le toliko optimalnih semenčic, kolikor je bilo na voljo jajčnih celic. Cilj novih laboratorijskih tehnik je bil usmerjen bolj v koncentriranje redkih, komaj opaznih semenčic v semenskem izlivu moških s težkimi oblikami oligo- ali kriptozoospermije. Od uvedbe metode ICSI je veljalo, da je za vnos v jajčno celico potrebno izbrati morfološko najoptimalnejši spermij. V primerih težkih oblik teratozoospermije je temu pogoju bilo težko zadostiti. Izkušnje pa so kmalu pokazale, da morfologija semenčic ne igra tako pomembne vloge pri oploditvi z metodo ICSI, kot jo ima sicer pri IVF in v pogojih in vivo, saj je mogoče zelo uspešno oploditi jajčne celice tudi s še ne popolnoma diferenciranimi semenčicami, dobljenimi iz tkiva mod.

V zadnjih letih se z razvojem optike pri mikroskopih uveljavljajo še nove metode za izolacijo morfološko najoptimalnejših semenčic. Z določenimi biotehnološkimi metodami pa je mogoče prepoznati tudi semenčice z nepoškodovano jedrno DNK.

V prispevku so naštete in opisane najpogosteje uporabljene metode za izolacijo semenčic iz različnih semenskih vzorcev in metode za selekcijo najprimernejših semenčic za oploditev jajčnih celic v postopkih OBMP.

Laboratorijske tehnike priprave semena za metode OBMP

Izbira semenčic za klasičen IVF postopek

Prvotni namen priprave semena bodisi za intrauterino inseminacijo ali in vitro fertilizacijo (IVF) je v glavnem temeljil na odstranitvi semenske plazme. Prvotna metoda je opisovala zgolj preprosto očiščenje semena. Del semenskega izliva je bilo potrebno razredčiti z gojiščem za celične kulture in epruveto z vzorcem centrifugirati. Za inseminacijo se je uporabilo usedlino iz negibljivih in gibljivih semenčic, poleg tega pa tudi ostalih celic in debrisa (Edwards in sod., 1969; Lopata in sod., 1978).

Za izbor samo gibljivih semenčic iz sedimenta so kasneje razvili metodo »swim-up«, ki je še danes najpogosteje v uporabi (Mahadevan in Baker, 1984). Metoda izhaja iz prejšnje. Po centrifugiranju se supernatant, ki vsebuje semensko plazmo, zavrže. Nad sediment centrifugiranega semena je potrebno počasi doliti nekaj svežega gojišča in epruveto inkubirati za pol ure. Ker gibljive semenčice izplavajo v višjo plast svežega gojišča (swim-up), je za klinično uporabo primeren ravno ta sloj. Metoda je učinkovita pri normozoospermiji in lažjih oblikah oligozoospermije, medtem ko pri astenozoospermiji in vzorcih s slabim utekočinjenjem semena v višje plasti izplava premajhno število semenčic. Večina jih ostane ujetih v zbitem sedimentu (**Slika 1 - v dodatku**).

Da bi se izognili centrifugiranju, ki lahko poškoduje semenčice ali pa še dodatno poslabša njihovo gibljivost, so poskušali s preprostejšo metodo »underlay«, pri kateri se na dno epruvete z gojiščem previdno odloži nekaj nativnega semena (Yovich in Stanger, 1984). Semenčice pri tej tehniki izplavajo v višje plasti čistega gojišča, vendar je možnost kontaminacije vzorca s semensko plazmo zelo visoka. Tej metodi so nekateri prilagodili tudi posebne epruvete z oddvojenim prostorom, kamor bi naj priplavale gibljive semenčice (angl. self-migratory method) (Makler in sod., 1984), vendar se ta tehnika v praksi ni ohranila.

Sčasoma se je pojavila potreba po izdatnejšem čiščenju semenčic in izolaciji čim večjega števila morfološko normalnih oblik. Istočasno pa so poskušali preprečiti koncentriranje vseh celic iz semenskega izliva v sedimentu. Slabost tehnike »swim-up« je ravno ta, da so semenčice dolgo v sedimentu in to v stiku z mrtvimi spermiji in levkociti ter so tako izpostavljene škodljivemu delovanju prostih kisikovih radikalov (Aitken in Clarkson, 1988). Zato so iskali bolj viskozne medije. Centrifugiranje semena skozi različno goste frakcije medija so povzročile selekcijo semenčic. V najgostejšo frakcijo so uspele priti večinoma semenčice bolj normalnih oblik. Te semenčice so bile tudi bolj izdatno očiščene od bakterij in debrisa. Tehnika se uporablja še danes in se imenuje centrifugiranje skozi viskozni gradient. Ločita pa se metoda kontinuiranega (Bolton in Braude, 1984) in nekontinuiranega viskoznega gradienta (Pousette in sod., 1986). Večjo gostoto medijev se je dobilo z uporabo različnih silika-gel medijev. Uporabljalo se je Ficoll® (Bongso in sod., 1989), Nycodenz in Percoll® (Gellert-Mortimer in sod., 1988).

Poleg naštetih tehnik je bila kratek čas v uporabi tudi metoda centrifugiranja semena skozi kolono steklenih vlaken (angl. glass wool column) (Paulson in Polakoski, 1977). Nastajale so tudi številne druge različice metod, s katerimi so poskušali reševati predvsem semenske vzorce z nizkim številom semenčic pri težjih oblikah moške neplodnosti. Med njimi sta dve, ki smo ju svoj čas uporabljali tudi v Sloveniji, in sicer IVF v mikrokapljici (angl. microdrop insemination) (Svalandeer in sod., 1994), ali uporaba dveh medsebojno povezanih kapljic, med katerimi so v eni bile jajčne celice, v drugi pa redke semenčice skupaj z ostalimi celicami in nečistočami. Gibljive semenčice naj bi preko ozkega kanalčka medija pripotovale v kapljico z jajčnimi celicami (angl. swim across) (Giorgetti in sod., 1992). Učinkovitost teh metod je bila slaba in rezultat je bil večinoma izostanek oplojenosti jajčnih celic.

Znani so še poskusi uporabe dodatkov semenu, ki bi naj izboljšali gibljivost semenčic, kot so: kafein (Garbers in sod., 1971), kalikrein (Gerhard in sod., 1990), 2-deoksiadenozin (Aitken in sod., 1986) in pentoksifilin (Rees in sod., 1990). Po objavi Yovicha in sod. (1990), ki je poročal o uspešnih IVF pri težji oligo- in astenozoospermiji, če je za pripravo semena uporabil pentoksifilin – stimulator gibanja semenčic, se je njegova uporaba hitro razširila.

Kljub temu je ostajalo precej primerov moške neplodnosti nerešljivih.

Danes še ni enotnega stališča, kdaj je semenski vzorec še primeren za klasično IVF metodo in kdaj za ICSI. Velja le, da je za klasičen IVF potrebno jajčne celice osemeniti s približno 100.000 do 200.000 dobro gibljivimi semenčicami v 1 mL (Bourne in sod., 2004). Če upoštevamo še morfologijo semenčic po strogih Tygerbergovih kriterijih, potem imajo moški z manj kot 5% normalnih semenčic slabše možnosti za starševstvo (59,3% stopnja oplojenosti in 15,2% stopnja zanositve), tisti s 5 do 14% normalnih semenčic boljše možnosti (77,6% stopnja oplojenosti; 26% zanositev), medtem ko se spermiogram z več kot 14% morfološko idealnimi semenčicami pojmuje kot normalen (Coetzee in sod., 1998).

Priprava in izbira semenčic za ICSI (Slika 2 - v dodatku)

Leta 1991 se je pri moških s slabšim spermiogramom začela uporabljati nova metoda ICSI. Ker se v začetnem obdobju ICSI še ni vedelo, kakšne stranske učinke ima lahko ta metoda na kasnejši razvoj otrok, je bila sprva namenjena le težkim oblikam moške neplodnosti. Iz semenskega izliva je bilo potrebno pridobiti toliko semenčic, kolikor je bilo na voljo jajčnih celic. Za upočasnitev gibanja semenčic pred posrkanjem v kapilaro in za dodatno očiščenje semenčic se je priporočala uporaba viskoznega polivinil pirolidona (PVP). Zaradi pomislekov o možnih toksičnih učinkih PVP na jajčno celico nekateri še danes ne odobravajo uporabe PVP in za injiciranje semenčice v jajčno celico uporabljajo kar navadno gojišče za semenčice. Seveda je pri slednjem postopku zaradi hitrega gibanja semenčice težje odkriti tiste z normalno morfologijo.

Semenski izliv moških s težko oligoastenoterato- ali kriptozoospermijo

Cilj novih laboratorijskih tehnik je bil usmerjen bolj v koncentriranje redkih, komaj opaznih semenčic v semenskem izlivu moških s težkimi oblikami oligoasteno- ali kriptozoos-

permije. Večinoma je bilo potrebno uporabiti celoten semenski vzorec, ga centrifugirati, in v sedimentu iskati morebitne semenčice. Zaradi pomislekov o možnem vnosu še drugega biološkega materiala (celični debris, bakterije, eksogena DNK, ipd.) v jajčno celico, se je za čiščenje semenčic priporočala uporaba gradient ali mini-gradient tehnike. Dobljeni sediment po centrifugiranju celotnega ejakulata se je nanesel nad gradient viskoznih tekočin v mikroepruveti in ponovno centrifugiral. Ker pogosto po uporabi te tehnike ni bilo mogoče najti semenčic, se je le-te iskalo v prvotnem sedimentu.

Od uvedbe metode ICSI je veljalo, da je za vnos v jajčno celico potrebno izbrati morfološko najoptimalnejši spermij. V primerih težkih oblik teratozoospermije je bilo temu pogoju težko zadostiti. Izkušnje pa so kmalu pokazale, da gibljivost, število in morfologija semenčic ne igrajo tako pomembne vloge pri oploditvi z metodo ICSI, kot jo imajo sicer pri IVF in v pogojih in vivo (Nagy in sod., 1998). Kljub temu embriologi danes porabijo precej časa, da med najdenimi semenčicami izberejo tiste, ki kar najbolje ustrezajo kriterijem normalne semenčice.

Pri uporabi PVP se semenski vzorec nanese v center kapljice PVP. Zaradi gostote PVP se dobro gibljive semenčice selekcionirajo same: iz centra PVP plavajo proti periferiji ter se kopičijo na robu kapljice, kjer pri večji povečavi (300 do 400X) lažje ocenimo morfološke dejavnike.

Aspirat ali biopsija testisa

Z uporabo ICSI je postalo mogoče zdraviti tudi moške z azoospermijo. Njihove semenčice lahko pridobimo z aspiracijo nadmodka ali moda bodisi z aspiracijo (angl. fine needle biopsy ali testicular sperm aspiration – TESA) (Silber in sod., 1994), bodisi z biopsijo moda (angl. testicular sperm extraction – TESE) (Schoysman in sod., 1993). Pogosto je semenčice potrebno izolirati iz zamrznjenega in odmrznjenega vzorca biopsije testisa. Semenčice iz testisa so zelo slabo gibljive ali celo negibljive, zato je njihovo iskanje v suspenziji testikularnih celic in eritrocitov zelo oteženo. Njihova izolacija mora biti opravljena mehansko z mikropipeto in uporabo mikromanipulatorja. V primerih neobstruktivne azoospermije, kjer gre za težje okvare spermatogeneze ali ohranitev spermatogeneze samo v določenem delu testisa, ni zagotovila, da bodo semenčice za osemenitev jajčnih celic tudi najdene, čeprav so bile v času histološke analize dokazane. Prav zaradi vsega tega je identifikacija in izolacija semenčic iz vzorca testisa med najtežjimi in časovno najbolj zamudnimi postopki OBMP.

Med postopki za obdelavo testikularnega tkiva ali aspirata je opisana večinoma le ena metoda, ki velja od začetnega obdobja TESE do danes. Košček tkiva testisa se razkosa na čim manjše delčke in s pomočjo skalpelov iz semenskih kanalčkov iztisne vsebino v obdajajoče gojišče. Večje delce se nato odstrani, preostalo celično suspenzijo pa očisti s centrifugiranjem skozi viskozni gradient. Spermije se išče v sedimentu (Verheyen in sod., 1995). Isti avtorji navajajo še možne različice te metode z uporabo mini-gradient tehnike ali uporabo pufra za lezijo eritrocitov, ki odstrani eritrocite – najpogostejše celice v celični suspenziji – in omogoči boljšo opaznost spermijev.

V literaturi je mogoče najti še opise bolj zahtevnih metod izdvajanja semenčic iz suspenzije celic testisa s pomočjo celičnih separatorjev (Aslam in sod., 1998; von Schonfeldt in sod., 1999).

Za primere, ko v testikularnem vzorcu ni mogoče najti gibljivih semenčic, so opisane naslednje možne tehnike: prva je podaljšano gojenje testikularnih celic in vitro (Edirisinghe in sod., 1996), druga pa dodajanje pentoksifilina, ki zelo učinkovito izboljša gibljivost semenčic (Kovacic in sod., 2006). Semenčice, dobljene neposredno iz moda, so pogosto slabo gibljive ali celo negibljive. Zamrzovanje testikularnega vzorca pa še dodatno poslabša že tako slabo gibljivost semenčic. Negibljive semenčice so lahko tudi mrtve, še posebno po odmrznjenju tkiva moda. V mnogih raziskavah je bilo potrjeno, da se z izbiro gibljivih semenčic, čeprav je iskanje lahko zelo zamudno, znatno poveča tudi uspešnost TESE-ICSI (Nagy in sod., 1998; Park in sod., 2003; Kovacic in sod., 2006). Pri tem zadošča, da semenčice kažejo vsaj neznatne premike repa, po čemer vemo, da so žive.

Vzorci s popolnoma negibljivimi semenčicami

Pri vzorcih s popolnoma negibljivimi semenčicami obstaja nevarnost, da so med izbranimi za ICSI večinoma mrtve semenčice. Razlogov za takšno stanje je lahko več. V kolikor je prisotno vnetje, gre lahko res za vzorec z mrtvimi celicami (nekrozoospermija). Lahko pa je razlog okvara spermatogeneze in nepopolno dozorevanje spermijev ali pa popolna odsotnost gibanja cilij (Kartagenerjev sindrom). Izbiro živih semenčic je mogoče opraviti, če vzorcu dodamo stimulator gibanja (npr. pentoksifilin) ali pa semenčice izpostavimo hipoozmotskemu mediju, ki pri živih semenčicah povzroči nabreklost repka (hipoozmotski ali HOS test). V literaturi je kar nekaj poročil o uspešnih ICSI postopkih, ko so semenčice za ICSI izbirali na ta način (Casper in sod., 1996; El-Nour in sod., 2001; Westlander in sod., 2003; Čižek-Sajko in sod., 2004).

Sodobnejše tehnike izbire semenčice za ICSI

Injiciranje izbranih morfološko optimalnih semenčic (IMSI)

V zadnjem času se je zaradi razvoja optičnih sistemov razvila možnost bolj natančnega opazovanja glavice semenčic in odkrivanje prisotnosti vakuol (Bartoov in sod., 2002). Metoda temelji na dobri optiki, ki daje bolj jasno globinsko ločljivost pri večji povečavi (6000 X do 12.500 X) kot je bilo možno z navadnim optičnim mikroskopom, kar omogoča izbor morfološko bolj normalnih semenčic za postopek ICSI (angl. intracytoplasmic morphologically selected sperm injection – IMSI). Vakuole naj ne bi vplivale na delež oplojenosti jajčnih celic, medtem ko bi naj bila povečana možnost za spontane splave (Berkovitz in sod., 2006).

Injiciranje akrosomsko aktiviranih semenčic

Akrosomsko aktivirane semenčice lomijo polarizirano svetlobo (angl. birefrigence), zato na polarizacijskem mikroskopu lahko ločimo semenčice, ki so sprostile vsebino akrosoma od tistih, ki akrosomske reakcije še niso imele. Dokazano je, da ICSI z akrosomsko reagiranimi

semenčicami daje boljše klinične rezultate v primerjavi z neaktiviranimi semenčicami (Gianaroli in sod., 2008).

Injiciranje semenčic, izbranih s hialuronsko kislino (HA)

Prisotnost receptorjev za hialuronsko kislino (angl. hyaluronic acid) na plazma membrani semenčic je v korelaciji z zrelostjo semenčice in integriteto njene DNK. S posebej oblikovanimi preparati in petrijevkami je mogoče doseči, da se semenčice s HA receptorji vežejo na HA na petrijevki. Tako lahko za ICSI uporabimo semenčice, ki imajo manj napak DNK (Jakab in sod., 2005; Huszar in sod., 2007; Parmegiani in sod., 2009).

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Vpliv okolja na kakovost semena

Impact of the environment on semen quality

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Povzetek

Slaba kakovost semena, rak moda, kriptorhizem in hipospadija so drug drugemu dejavniki tveganja, prav tako pa domnevajo, da vse štiri bolezenske entitete izvirajo iz testikularne disgenezije. Pri najblažji obliki testikularne disgenezije je lahko edini simptom nekoliko zmanjšana kakovost semena, medtem ko so pri najhujši obliki prisotni vsi štirje znaki. Te štiri bolezni so v zadnjih desetletjih postale bolj pogoste, vsaj v Severni Evropi. Genetski dejavniki so lahko vzrok majhnemu deležu primerov, medtem ko se endokrini motilci iz okolja vse bolj kažejo kot možen vzrok porasta pojavnosti v zadnjem času. Zato preiskujejo kemikalije, kot so ftalati, zaradi splošne izpostavljeni človeka le-tem, in ker pri živalih učinkujejo anti-androgensko, vendar ftalati sami ne morejo biti vzrok za toliko primerov težav z moškim reproduktivnim zdravjem. Bolj verjetno je, da gre za skupni učinek številnih endokrinih motilcev, ki so prisotni v nizkih količinah v okolju. Okoljski dejavniki lahko vplivjo na testikularni razvoj v maternici, vendar verjamemo, da obstajajo tudi neposredni negativni učinki na odrasli testis. Tega koncepta še niso veliko preučevali, vendar je naša skupina pred kratkim dokazala, da so visoke serumske vrednosti perfluoroalkilne kisline pri mladih moških povezane z znižanim številom morfološko normalnih spermijev.

Abstract

Impaired semen quality, testicular cancer, cryptorchidism and hypospadias are risk factors for each other, and it has been suggested that all four disease entities may result from testicular dysgenesis. In the mildest form of testicular dysgenesis the only symptom may be a slightly reduced semen quality, whereas the most severe cases are afflicted with all four

symptoms. The four diseases have, at least in the Northern European countries, become more frequent during the last decades. Genetic factors can lead to some cases of testicular dysgenesis, but endocrine disruptors in the environment are emerging as possible causative factors for the recent increase in frequency. Chemicals such as phthalates are being investigated because of universal human exposure and anti-androgenic effects in animals, but phthalates alone cannot be responsible for the many cases of problems with male reproductive health. Mixture effects of low-dose exposure to several endocrine disrupting chemicals in the environment must be considered as a more likely explanation. Environmental exposures may have an effect on testicular development in utero, but we believe that there may also be adverse effects on the adult testis directly. This concept has been much less studied, but we have recently shown that high serum levels of perfluoroalkyl acids in young men are associated with decreased number of morphologically normal spermatozoa.

Key words: Testicular dysgenesis syndrome, semen quality, testis cancer, cryptorchidism, hypospadias, endocrine disruptors.

Ključne besede: Sindrom testikularne disgeneze, kakovost semena, rak moda, kriptorhizem, hipospadija, endokrini motilci.

Introduction

As testicular cancer, hypospadias, cryptorchidism and hypospadias seem to become more common in some countries, it is becoming clear that genetic changes cannot serve as an explanation for the increasing frequency of these conditions. Changes in our environment should, in our view, be considered as a more plausible explanation, and knowledge of specific factors with adverse effects on male reproductive health must be sought. Epidemiological studies of patients with testicular cancer point to the importance of the prenatal environment, but we believe exposures in adult life may also have significant effects on testicular function. The following will provide a brief overview of this topic.

Testicular Dysgenesis Syndrome

The rapidly rising incidence in several European countries of conditions relating to male reproductive health has led to the belief that such conditions may have a common origin (Skakkebæk *et al.,* 2007). The concept of the Testicular Dysgenesis Syndrome (TDS) was first proposed in 2001 (Skakkebæk *et al.,* 2001), when it was hypothesized that many cases of poor semen quality, testicular cancer, cryptorchidism, hypospadias and certain disorders of sex development, may all be the result of a common developmental disorder originating in early fetal life.

Clinically, patients can present TDS symptoms with varying severity: mild cases with slightly impaired spermatogenesis and severe cases with all of the above mentioned conditions, including testicular cancer. The clinical problems comprised in TDS are risk factors for each other, and it is now known that some men with severely decreased semen quality have an increased risk of testicular cancer as well as its preinvasive precursor, carcinoma in

situ (CIS) (Berthelsen 1987; Møller and Skakkebæk, 1999; Baker *et al.,* 2005; Mancini *et al.,* 2007).

We do not know the prevalence of TDS, but the incidence of testicular cancer has risen several times over the past 70 years and is now the most common malignancy among young men in Denmark. Recent estimates indicate that 5–9% of the Danish male population may suffer from undescended testes, hypospadias or testicular cancer (Boisen *et al.,* 2004; Boisen *et al.,* 2005). Up to 40% of healthy young Danish men from the general population have decreased semen quality (Jørgensen *et al.,* 2006). The median sperm concentration among young men in Denmark is approaching $40x10^6$/mL, a level under which the probability of pregnancy per menstrual cycle may markedly decrease (Bonde *et al.,* 1998). Some mildly affected men may have no clinical symptoms at all, as even severely decreased spermatogenesis is sometimes compatible with fertility. Taking into account the high frequency of low sperm counts, TDS related symptoms seem to be quite common in Denmark. In many countries, there is a growing need for assisted reproduction (Nyboe and Erb, 2006), and although declining semen quality is only part of the reason for this problem, it seems as if semen quality in some countries may be reaching a point where any further decline may have serious consequences for human fertility (Andersson *et al.,* 2008). We regard the increasing frequency of patients with TDS related symptoms as real, and not attributable to changes in reporting or diagnosis of the symptoms.

Another emerging aspect of testicular dysgenesis is that men with poor semen quality may have impaired Leydig cell function. A large study comparing reproductive hormone levels in infertile men (with severe abnormalities of sperm production) to those of fertile controls revealed lower testosterone levels, lower testosterone/LH ratios and higher estradiol/testosterone ratios (Andersson *et al.,* 2004). Adults with testicular cancer also seem to have signs of decreased Leydig cell function, as well as some boys with hypospadias (Rey *et al.,* 2005) and some newborn boys with cryptorchidism (Suomi *et al.,* 2006). This provides some indirect evidence suggesting an early developmental origin. It has been known for many years that a man's serum testosterone levels decreases with age. Observational studies of sex hormone levels in US and Danish population suggested an age-independent decrease in testosterone levels over the last 20 years that cannot be fully explained by health and lifestyle factors (Travison *et al.,* Andersson *et al.,* 2007). The reason for this is not known, but again it seems obvious to search among environmental factors, with endocrine disrupting chemicals as candidates.

Clearly, not all cases of poor semen quality can be explained by TDS. Obstructive azoospermia, varicocele, occupational exposures to toxic compound or treatment with chemotherapeutic agents or radiation exemplify this. Rarely, the explanation can be found in chromosomal aberrations (e.g. Klinefelter syndrome) or other genetic causes, such as microdeletions of the Y chromosome (Vogt, 2005). In a clinical setting, however, the cause for decreased semen quality is most often unclear, and we believe that most of these unexplained cases may be due to environmental factors, in early fetal life, as well as a contribution from later exposures.

Hypospadias, as well, cannot always be associated with TDS, as some cases are seen in men with normally developed testes (Asklund *et al.,* 2009). On the other hand, it is believed that all testicular germ cell cancers have a prenatal origin in a dysgenetic testis, being preceded by carcinoma in situ, precursor cells. The majority of cryptorchidism cases, as well, are most likely linked to TDS (Rajpert-De Meyts, 2006).

Prenatal exposure to endocrine disruptors

In the past few years, exposure to endocrine-disrupting compounds has been suggested to play a role in the increased numbers of patients with TDS-like symptoms. Evidence from animal models (Sharpe, 2006), as well as epidemiological research in humans, points to chemicals that are commonly used and ubiquitous in our environment. Such environmental factors are, in our view, the most important determinants of TDS.

Several groups of chemicals (e.g. phthalates and persistent pesticides) are now suspected of anti-androgenic or estrogenic effects that may possibly affect semen quality. Animal studies have shown that such endocrine disrupting chemicals can cause TDS-like symptoms when administered in early fetal life. *In utero* exposure of rats to certain phthalates such as di(n-butyl)phthalate (DBP), results in a range of dysgenetic features in the male offspring with many similarities to TDS in humans. This spectrum of disorders in rats has been termed the "phthalate syndrome", first coined in 2003 (Gray Jr and Foster, 2003). Humans are exposed to considerable amounts of these same chemicals from a range of consumer products (Blount *et al.,* 2000; Frederiksen *et al.,* 2007), and therefore it seems plausible that exposure to phthalates could be involved in TDS in humans. Phthalates, however, are only one group of chemicals that are suspected of endocrine disrupting effects. Other candidates, including perfluoroalkyl compounds, pesticides and Bisphenol A are other currently interesting candidates. Furthermore, the importance of mixture effects of low-dose exposure to multiple compounds is becoming clear from animal studies, and must be considered when piecing together information from epidemiological studies to come (Hass *et al*., 2007; Kortenkamp *et al*., 2007).

Causal links in humans, however, are difficult to establish, and we must rely on epidemiological studies to find plausible candidate exposures. A study of *in utero* exposure to smoking and semen quality among 1770 young men from 5 European countries (Jensen *et al.,* 2004) supports the TDS hypothesis, together with epidemiological studies suggesting an association between early phthalate exposure and male reproductive health symptoms, including maternal phthalate exposure during pregnancy and decreased ano-genital length in male infants (Swan *et al.,* 2005) and phthalates in breast milk and changes in the pituitary-gonadal axis in male offspring (Main *et al.,* 2006).

Even if studies of isolated chemicals may show no significant results, it is possible that mixtures of several chemicals in our surroundings may be significant, even if the effects of the chemicals separately are obscured. There is, therefore, a scarcity of human data that can justify disuse of specific endocrine disruptors. Exposure to environmental factors is extremely complex, but the global exposure to endocrine disruptors raises concern for effects on not just fertility but also metabolic syndrome, early puberty and genital malformations.

Other environmental factors than endocrine disrupting compounds may contribute to declining semen quality. Obesity is a life-style factor associated with decreased semen quality (Jensen *et al.,* 2004), although it is not known whether infertile men are predisposed to obesity, or whether obesity can contribute causally to male infertility.

Exposures of adult men

It is known that some chemical substances may have adverse effects on male fertility by exposure in adulthood. Lead and cadmium, for instance, accumulate in the male reproductive organs, and high exposure to these substances has been associated with reduced semen quality (Abadin *et al.,* 1997). Another example is the pesticide 1,2-dibromo-3-chloropropane (DBCP), which permanently impaired spermatogenesis in exposed male workers (Whorton and Meyer, 1984). Such occupational exposures, evidently, are not related to the gonadal development, and are thus cannot be considered as a part of TDS.

Increasing attention, however, is being paid to the possibility that endocrine disrupting compounds may not only have an affect in early fetal life, but that exposures later in life may contribute to impairment of semen quality. Furthermore, we cannot rule out that some men, e.g. men born with cryptorchidism or men with varicocele, may be more susceptible to environmental exposures in adulthood.

Perfluoroalkyl acids (PFAAs) may be an example of such a current exposure. PFAAs are degradation products from the production of many consumer and industrial products, for example, for impregnation of carpets, textiles, and paper (Jensen *et al.*, 2008; Jensen and Leffers, 2008; Kissa, 2001). In animal studies, perfluorooctanoate (PFOS) exposure has been shown to reduce bodyweight, induce changes in the liver, alter levels of thyroid and reproductive hormones, and induce Leydig cell hyperplasia and/or adenomas in the testes of exposed animals (Cook *et al.,* 1992; Biegel *et al.,* 1995). This led to suspicion that PFAAs could act as endocrine disruptors in humans as well, and a pilot study from the Department of Growth and Reproduction has just been published as the first ever to indicate a correlation between semen quality and PFAAs (Joensen *et al.,* 2009). We found that the men with highest levels of PFOS and PFAS had 60% fewer morphologically normal sperm than the men with the lowest levels. This large difference found in the pilot study is alarming, and if the findings from this preliminary study can be corroborated, high levels of PFAAs may contribute to the otherwise unexplained low semen quality seen in many young men. The study could not prove an association between PFAA levels and reproductive hormones, and larger follow-up studies are needed to clarify this issue. Very few studies of other endocrine disruptors (e.g., phthalates, pesticides) have shown association with impaired semen quality (Duty *et al.*, 2003; Meeker *et al.*, 2008; Swan *et al.*, 2003).

Conclusion

In the last few decades, there has probably been a steep increase in male reproductive health problems, and we are only now beginning to see the consequences. Endocrine disrupting compounds that are ubiquitous in our environment are suspected to be at least partly responsible, but as long as we do not know specifically which chemicals, or mixtures of

chemicals, are harmful to human fertility, it is difficult to commence preventive strategies. Considering the currently limited evidence from human studies, further information on the reasons for critically low semen quality in men from many countries is urgently needed.

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Endokrini motilci

Endocrine disruptors

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Povzetek

Hormonski ali endokrini motilci so različne snovi, ki se nahajajo v našem okolju in lahko motijo razvoj in delovanje endokrinega sistema. Verjetno najbolj proučevani hormonski motilci so ftalati in bisfenoli, čeprav se je ugotovilo tudi za številne druge kemične snovi, da lahko motijo delovanje endokrinega sistema ali z motenjem delovanja endogenih hormonov ali pa s posnemanjem njihovega delovanja. Atrazin je široko uporabljan herbicid in eden od najpomembnejših onesnaževalcev vode. V naši raziskavi smo preverjali, ali lahko izpostavljenost atrazinu v koncentracijah, pomembnih za izpostavljenost splošne populacije ljudi, vpliva na razvoj spolnega sistema pri mišjih samcih. Mišji pari so prejemali 0.5 µg atrazina/L pitne vode 1 teden pred parjenjem, med parjenjem in brejostjo do odstavitve mladičev 3 tedne po skotitvi. Razvoj mod smo spremljali pri potomcih tretiranih miši, ki so bili izpostavljeni atrazinu samo preko mater (v maternici in preko mleka) do odstavitve pri treh tednih starosti. Samice, ki so prejemale v pitni vodi atrazin so imele statistično značilno manjše število mladičev v gnezdu (7,06) v primerjavi s samicami iz kontrolne skupine (5,06; $P< 0{,}01$). V modih mladih samcev smo ugotovili močnejšo izraženost anti-Müllerjevega hormona (AMH) pri samcih, ki so bili preko mater izpostavljeni atrazinu. Število apoptotičnih spolnih celic je bilo statistično zanesljivo povišano pri mladičih zdravljenih mater pri starosti 48 dni, medtem ko je bila pri starosti 70 dni dnevna produkcija semenčic statistično zanesljivo nižja v skupini, ki je bila v času do odstavitve preko mater izpostavljena atrazinu. Rezultati naše raziskave tako kažejo, da ima izpostavljenost nizkim odmerkom atrazina v neonatalnem obdobju pomemben dolgotrajen vpliv na razvoj spolnega sistema pri samcih.

Abstract

Endocrine disruptors are chemicals released into the environment that have the capacity to affect the development and function of the endocrine system. The most studied endocrine disruptors are probably phthalates and bisphenols, although many other chemicals have been shown to interact with the endocrine system either by mimicking hormone action or interfering with endogenous hormones. Atrazine is one of the most widely used herbicides and one of the most important water pollutants. In our study we examined whether exposure to low doses of atrazine (relevant for general human exposure) through drinking water affects neonatal development of the male reproductive tract in mice. Breeding pairs of mice received 0.5 μg of atrazine/L drinking water for one week before mating, during mating and until weaning at three weeks of age. Testis development was examined in offspring of treated mice, which were exposed to atrazine *in utero* and through milk, but not after weaning. Atrazine-treated mothers had significantly smaller litter sizes in comparison to the control group (7.06 versus 5.06 pups per litter, $P< 0.01$). In young testes, the expression of anti-Müllerian hormone (AMH) at 9 days of age was significantly increased in offspring of atrazine-treated mothers. The number of apoptotic germ cells was significantly increased in the testes from atrazine-exposed mice at 48 days of age while daily sperm production was significantly decreased in atrazine-exposed group at 70 days of age, suggesting that neonatal and early postnatal exposure to low levels of atrazine has long lasting deleterious effects on testis development.

Ključne besede: Anti-Müllerjev hormon, apoptoza, atrazin, endokrini motilci, herbicid, miške, moški reproduktivni trakt.

Key words: Anti-Müllerian hormone, apoptosis, atrazine, endocrine disruptors, herbicide, mice, male reproductive tract.

Introduction

Endocrine disruptors are chemicals present in the environment that have the capacity to interfere with the endocrine system. Many in vivo and in vitro studies have shown that chemicals such as phthalates, bisphenols, PCBs and DDT derivatives could mimic hormone action thus interfering with hormonal homeostasis. Atrazine is one of the most commonly used herbicides in the world and a very common contaminant of ground and underground water. Several studies have shown that atrazine could affect the endocrine system, primarily the hypothalamic-pituitary-gonadal axis (Wetzel *et al.*, 1994; Cooper *et al.*, 1996; Eldridge *et al.*, 1999; Ashby *et al.*, 2002). Some studies in male rats demonstrated that atrazine could also affect the male reproductive system. Two studies reported that the treatment of rats with atrazine during puberty caused a significant reduction in both intratesticular and serum testosterone levels, and reduced weights of prostates, seminal vesicles and epididymes. However, it is not clear whether this reduction in the organ size was due to direct effects of atrazine or to reduced body weights in treated rats (Stoker *et al.*, 2000; Trentacoste *et al.*, 2001). Recent studies have shown that *in utero* and through milk exposure atrazine affects the development of prostate and seminal vesicles, suggesting that atrazine can pass through the placenta and/or into milk and can affect offspring of treated

mothers (Rayner *et al.*, 2007). However, almost all studies examining atrazine effects in mammals used large doses of atrazine, which could mimic an exposure of workers working with atrazine, but are usually not a concern for the general population. However, there are no studies reporting whether exposure to atrazine *in utero* and during the early postnatal period, a critical period for male reproductive tract development, could affect testis development also at doses that could be found in drinking water and could therefore be a concern for public health.

Material and methods

Animals

Sexually mature (60-70 days old) BALB/c male and female mice were exposed to 0.5 μg of atrazine per L via drinking water. Male and female mice were treated with atrazine for one week separately, after which one male and one female mouse were joined in a single cage. Mice were exposed to atrazine throughout pregnancy and lactation period until weaning at 21 days. Litter size was carefully monitored; pups were always counted on the day of delivery. Male pups from different litters were randomly assigned to groups for sacrifice at different postnatal age. Pups were sacrificed at 9, 48 and 70 days of age, 48 and 70 days old mice were exposed to atrazine only until weaning on day 21. Nine-day and 70-day old mice were sacrificed by CO_2 exposure followed by cervical dislocation. 48-day old mice were anaesthetized and perfused with Bouin's solution. Testes were isolated and postfixed in Bouin's solution. Subsequently, the testes were processed into paraffin wax. Animal experiments were approved by the Veterinary Commission of Slovenia and were done according to the EU directive and NIH guidelines.

Daily sperm production

Daily sperm production was measured as described before (Thayer *et al.*, 2001). Briefly, 70-day old male mice were euthanized, the testes were removed and weighed, and then homogenized in 5 ml of physiological saline containing 0.05% (v/v) Triton X-100. One drop of the homogenizate was transferred into Bürker-Türk counting chamber. Elongated spermatids were counted twice in 10 visual fields under magnification 400x. Daily sperm production per gram of testis tissue was calculated using a formula: (N x 125000)/weight of testes where N is the number of counted spermatids (average from two countings) and 125000 a dilution factor. Developing spermatids spend 4.84 days in steps 14–16 during spermatogenesis in the mouse. Thus, the values for the number of spermatids per testis and spermatids per gram of testis were divided by 4.84 to obtain a daily sperm production and efficiency of sperm production (per gram of testis).

Immunohistochemistry and apoptosis detection

Immunocytochemistry was performed as described before (Majdic *et al.*, 1997). Specific primary antibodies directed against AMH (gift from Dr Nathalie Josso, France) were used at a dilution of 1:100 and polyclonal rabbit antibodies against 3 -HSD (gift from Dr Ian

Mason, Edinburgh, Scotland) were used at a dilution of 1:500. Specificity of the antibodies was controlled by using non-immune rabbit serum instead of primary antibodies. Apoptotic cells were visualized using Chemcon ApopTaq peroxidase kit (Millipore, Billerica, MA, USA) following the manufacturer's instructions.

AMH measurements

Blood was collected from 9-day old mice at the time of sacrifice through cardiac puncture. Heparinized blood was centrifuged at 2000 rpm for 3 minutes. Plasma was removed and stored at -20°C until use. AMH serum levels were measured with an in house AMH ELISA assay (Kevenaar *et al.*, 2006; Visser *et al.*, 2007).

Statistical analyses

In all analyses, at least five, but usually more, samples per group were examined and mice examined were always from at least three different litters for each age group. NCSS software package (NCSS, Kaysville, UT, USA) was used for all statistical analyses. One way ANOVA followed by student T-test was performed to determine whether there were significant differences between groups with $p<0.05$ considered as significantly different.

Results

Litter size

Litter size of mothers treated with atrazine was significantly reduced. In the control group, the average litter size was 7.06 ± 0.51 pups per litter (mean ± S:E.M., n=16) whereas in the atrazine-treated group the average litter size was 5.06 ± 0.50 pups per litter (mean ± S.E.M., n=16; $p < 0.01$). Throughout the experiments, body weight of mothers treated with atrazine was measured and there were no differences between control and atrazine-exposed mice suggesting that atrazine in water did not affect food or water consumption, and therefore the differences in body weights and/or food consumption could not affect litter size.

Daily sperm production

Daily sperm production per gram of testis tissue in 70-day old mice was significantly reduced in atrazine-exposed group in comparison to the control group with atrazine-exposed group having 31.660.534 ± 1.587.886 sperm per g testis and control group 39.386.616 ± 1.821.617 ($p < 0.01$).

Immunocytochemical staining and AMH blood levels

Immunocytochemical staining with antibodies against AMH on day 9 appeared stronger in the atrazine-treated group in comparison to the control group (**Fig. 1a** and **b - appendix**). The increase in AMH expression was also reflected by a significant increase in AMH serum levels with the atrazine-exposed group having 69.1 ± 7.7 ng/mL in comparison to the control group with 46.5 ± 4.8 ng/mL ($p < 0.05$).

Apoptosis

We examined the number of apoptotic cells in the testes of 48-day old mice and found a significant increase in the average number of apoptotic cells as detected by TUNEL assay in the group exposed neonatally to atrazine (59.7 ± 5.8 cells per testis section) in comparison to the control group (41.4 ± 2.5; $p < 0.05$).

Discussion

In the last 15 years, it has been demonstrated that many man-made chemicals could interact with the hormonal system either by mimicking hormone action or acting as agonists preventing hormones to act. These chemicals have been named endocrine disruptors and many studies have shown diverse effects of these chemicals and associated them with reproductive abnormalities, breast cancer, precocious puberty and even obesity. However, there are very few studies on endocrine disruptors that are directly relevant for human health and although many studies do suggest that some of these chemicals could pose a threat for human health, especially for developing fetuses, we do not have a conclusive answer whether endocrine disruptors at doses important for general human population could affect the development and/or function of the human endocrine system (Sharpe and Irvine, 2004). Atrazine is one of the most widely used herbicides and as such, a very common water pollutant. While WHO and EPA guidelines set safe levels of atrazine in drinking water at 2 µg/L and 3 µg/L, respectively, the EU limits for atrazine contamination are set at 0.1 µg/L of drinking water (EPA, 2006; Wenzel *et al.*, 2003; WHO, 2006). However, also in the EU, these levels were often breached and could reach up to 1 µg/L in the areas with intensive farming (Wenzel, Müller and Ternes, 2003). In our study, we examined whether exposure to atrazine *in utero* and neonataly via milk could affect male reproductive tract development. This is especially important as it is known that many aspects of testicular function in adult life are determined during the neonatal period (Sharpe *et al.*, 1995). We found that atrazine exposure caused a delay in postnatal testis development as demonstrated by a higher testicular expression and serum levels of AMH in exposed mice in comparison to control mice on day 9, and a higher ratio of seminiferous tubules without lumen on day 19 in atrazine-exposed mice in comparison to control mice (not shown). AMH is usually strongly expressed in Sertoli cells throughout fetal development, but its expression diminishes shortly after birth concurrently with Sertoli cell maturation (Josso *et al.*, Sharpe 2006). Increased expression of AMH on day 9 strongly suggests a delay in early maturation of Sertoli cells. It is known that the inhibition of AMH during puberty is androgen dependent (Rey *et al.*, 1993). Therefore, our findings could suggest diminished testosterone levels in atrazine-exposed mice at an earlier period, or, alternatively, it could also suggest a diminished testosterone action due to reduced activity of androgen receptors. Lower testosterone levels could be the result of atrazine-induced aromatase expression, leading to an increased testosterone conversion to estradiol, which had been suggested as a mode of atrazine action. Apoptosis in testicular germ cells is a normal process, usually more prominent during the first wave of spermatogenesis (Print and Loveland, 2000; Sinha Hikim *et al.*, 2003). However, an increase in the number of apoptotic cells is commonly seen in pathological situations and could be triggered by various factors such as exposure to differ-

ent chemicals or heat, or as a response to reduced levels of testosterone or FSH (Print and Loveland, 2000; Sinha Hikim, Lue, Diaz-Romero, Yen, Wang and Swerdloff, Dohle *et al.*, 2003; Meachem *et al.*, 2005). The first wave of spermatogenesis in mice is usually concluded by day 48. In our study, the testes from both control and atrazine-exposed mice showed a complete spermatogenesis on day 48, however, the number of apoptotic cells in the testes of atrazine-exposed mice was significantly increased. As exposure to atrazine in our experiment concluded on day 21, it is unlikely that the increased apoptosis was a direct effect of atrazine, although this option cannot be ruled out as atrazine is a fairly stable molecule, and could therefore still be present in the organism 30 days after exposure. Another possible explanation for the increased apoptosis could be reduced levels of testosterone, which had previously been connected with increased apoptosis (Dohle, Smit and Weber, 2003).

In conclusion, this study demonstrates that atrazine could act as an endocrine disruptor in mice even at very low doses, which is relevant for general human exposure, and that neonatal exposure to atrazine through mothers could have long lasting deleterious effects on testis development. This has consequences for the testicular function in adult life as demonstrated by reduced daily sperm production and increased number of apoptotic cells in adult offspring of exposed mice. Although at present the mechanisms that lead to these effects are not known, the results of this study present a concern for public health, especially since we have also detected a significantly reduced litter size in mice exposed to atrazine through drinking water.

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Okolje in rak moda

Environment and testis cancer

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Povzetek

Kljub nizki skupni incidenci raka mod (RM) (1 % vseh malignih neoplazij), je le-ta najpogostejši maligni rak med mladimi moškimi. V zadnjih 40 letih se je njegova incidenca v razvitih državah bistveno povečala (Bray in sod., 2006; Huyghe in sod., 2007). V ZDA se je skupna incidenca germinalnih tumorjev mod povečala za več kot 44 odstotkov, s 3.35/100.000 primerov na leto v obdobju med 1973 in 1978 na 4.84/100.000 primerov na leto v obdobju med 1994 do 1998. Porast je bil večji za seminome (64%) v primerjavi z neseminalnim germinalnim rakom mod (24%) in med belci v primerjavi s črnci (52% proti 25%). V raziskavi, narejeni v 12 evropskih državah (Češka Republika, Danska, Finska, Francija, Italija, Norveška, Slovenija, Slovaška, Španija, Švedska, Švica, Velika Britanija), so ugotovili porast incidence RM v obdobju med letoma 1983 in 1997 v vsaki od vključenih držav. V Veliki Britaniji se je podobno naraščajoči trend incidence RM odrazil v podvojitvi skupne incidence od 5-6/100000 na 10-11/100000 v med letoma 1974 in 1991 pri moških, starih med 20 in 49 let. Tudi v Franciji beležijo porast incidence RM, posebej v severovzhodnih predelih.

Vendar so v stopnji incidence RM v sosednjih državah opazne velike razlike. Primerjava med dvanajstimi evropskimi državami je pokazala 5-kraten odklon v incidenci, saj so se lete gibale od 3/100.000 v Španiji do 15/100.000 na Danskem in v Švici).

Etiologija RM ostaja še dokaj neznana.

Edina do sedaj dokazana dejavnika tveganja za RM sta kriptorhizem in v manjši meri tudi družinska obremenitev (McGlynn, 2001; Hemminki and Chen, 2006). Glede na druge

rake pri družinskih članih, je nekaj objavljenih raziskav, ki so ugotovile povezavo med RM in levkemijo, rakom debelega črevesja, dojk ter ledvic, melanomom, tumorji vezivnega tkiva, pljučnim rakom, ne-Hodgkinovim limfomom in Hodgkinovo boleznijo v družinski anamnezi (Hemminki and Li, 2004; Hemminki and Chen, 2006).

Čeprav so mnoge raziskave ugotovile povezavo med poklicem in RM, še vedno ni dokončnih zaključkov o tej povezavi. Vendar pa so ugotovili značilno povezavo z nastankom RM pri moških, izpostavljenim različnim okoljskim ali poklicnim dejavnikom, na primer pri strojilcih usnja (Levin in sod., 1987), mehanikih v letalstvu (Ryder in sod., 1997), policistih (Finkelstein, 1998), delavcih v industriji nafte in plina (Mills in sod., 1984), delavcih v tekstilni idustriji, izdelovalcih preprog (O'Brien and Decouflé, 1988), delavcih v papirni industriji (Andersson in sod., 2003), v industriji plastike in kovin (Rhomberg in sod., 1995) in pri kmetih, ki so izpostavljeni pesticidom (Fleming in sod., 1999). Posledično je nastal dolg, vendar z dokazi ne vedno podprt seznam molekul, snovi in topil, ki bi lahko delovali kot dejavniki tveganja za nastanek RM. V zadnji kontrolirani raziskavi (229 primerov in 800 kontrol iz bolnišničnega okolja) o vplivih družinske anamneze, okoljskih in poklicnih pogojih, so se kot dejavniki tveganja za nastanek RM izpostavili samo življenje v kmečkem okolju, kriptorhizem v anamnezi moškega, kriptorhizem v družinski anamnezi, RM in rak dojke (Walschaerts in sod., 2007).

Zaključek

Incidenca RM v razvitih državah narašča. Pomembni za klinično prakso so predvsem informiranje, odkrivanje in zgodnje zdravljenje RM.

Ugotovljen je bil vpliv letnice rojstva na spremembe v krivulji incidence RM. Berström *in sod*. (1996) so ugotovili, da je bilo relativno tveganje pri moških, rojenih okoli leta 1965 na Švedskem 3.9 (2.7-5.6), in pri moških, rojenih v Vzhodni Nemčiji 11.4 (8.3-15.5) glede na referenčne moške, rojene okoli 1905. Ob tem pa je več avtorjev ugotovilo pomembno zniževanje incidence RM pri moških, rojenih med drugo svetovno vojno. Vsa ta opažanja govorijo bolj v prid vplivom okolja kot vplivom genetike na razvoj RM.

Okoljski dejavniki/okoljska izpostavljenost imata ključno vlogo za nastanek RM, tako da sta posledično življenjski obdobji *in utero* in mogoče še v puberteti ključni odbobji za reproduktivno zdravje, ko lahko endokrini motilci in asimilati medsebojno vplivajo.

Abstract

Despite a low overall incidence of testicular cancer (1% of all malignant neoplasms), it is the most common malignancy among young men. Over the last 40 years, this incidence rate has substantially risen in most industrialised countries (Bray *et al.*, 2006; Huyghe *et al.*, 2007). In the United States, the overall incidence of TGCT rose by more than 44% from 3.35 per 100,000 person-years in 1973-1978 to 4.84 per 100,000 person-years in 1994-1998. Nevertheless, the rise was more pronounced for seminomas (64%) than for NSGCT (24%) and for white than for black men (52% versus 25%). In a study conducted in 12 European countries (Czech Republic, Denmark, Finland, France, Italy, Norway, Slovakia,

Slovenia, Spain, Sweden, Switzerland and the United Kingdom), an increase in testicular cancer incidence was observed in all of these countries during the period 1983-1997. In Great Britain, a similar increasing temporal trend in TC incidence resulted in a doubling of the overall incidence rate, rising from 5-6 per 100,000 to 10-11 per 100,000 between 1974 and 1991 in men aged 20-49. In France, short reports suggested that TC incidence rate has also risen, especially in the north-eastern regions.

We clearly also have discrepancies in TC incidence rates in neighboring countries. In a comparison between 12 European countries, there was a five-fold variation in incidence with rates ranging from 3 per 100,000 in Spain to 15 per 100,000 in Denmark and Switzerland.

So far, the aetiology of testicular cancer remains largely unknown.

Only cryptorchidism, and to a lesser extent a family history of testicular cancer, may be considered as well-established risk factors (McGlynn, 2001; Hemminki and Chen, 2006). Regarding other cancer in relatives, few papers showed association between leukaemia, distal colon and kidney cancer, melanoma, connective tissue tumours, lung cancer, breast cancer and non-Hodgkin's lymphoma and Hodgkin's disease in families and TC (Hemminki and Li, 2004; Hemminki and Chen, 2006).

Although many studies have found a positive association between occupation and testicular cancer, no definite conclusion has really emerged. Nevertheless, a significant relationship with the occurrence of testicular cancer has been observed in men exposed to various environmental or occupational conditions, such as leather tanners (Levin *et al.*, 1987), airframe repairers (Ryder *et al.*, 1997), policemen (Finkelstein, 1998), gas and petroleum workers (Mills *et al.*, 1984), carpet and textile workers (O'Brien and Decouflé, 1988), paper workers (Andersson *et al.*, 2003), plastic and metal workers (Rhomberg *et al.*, 1995), and pesticide-exposed farmers (Fleming *et al.*, 1999). Consequently, a long list of molecules, compounds and solvents has been suspected to be related to an excess risk of testicular cancer, but with lack of consistent evidence. In a recent hospital-based case-control study (229 cases and 800 controls) on familial, environmental and occupational conditions, only living in a rural area, a history of cryptorchidism in the men, cryptorchidism in relatives, testicular cancer, and breast cancer were significant TC risk factors (Walschaerts *et al.*, 2007).

In conclusion several points remain crucial:

TC incidence is increasing in industrialized countries - information, detection and early treatments still constitute objectives for clinicians especially those involved in reproductive health.

A clear birth cohort effect was observed in the changes in the slope of TC incidence rates. For example, using men born around 1905 as the reference group, Bergström *et al.* (1996) noted that the relative risk of having a TC in men born around 1965 was 3.9 (2.7-5.6) in Sweden and 11.4 (8.3-15.5) in East Germany. Furthermore, several authors have observed that the TC incidence rate markedly decreased in the cohort of men born during the Sec-

ond World War. All these observations are clearly in favor of an environmental impact rather that a genetic phenomenon.

Environmental conditions /exposure certainly play a key role in the occurrence of TC and consequently *in utero* life and possibly puberty period are certainly key reproductive health windows where endocrine disruptors and assimilates could interfere.

Key words: Birth cohort effect, cryptorchidism, environment, family history, increased incidence, occupation, rural area, seminoma, testicular cancer.

Ključne besede: Kohortni učinek letnice rojstva, kriptorhizem, okolje, družinska anamneza, povečana incidenca, poklic, podeželje, seminom, rak moda.

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Postopek klinične diagnoze v obravnavi neplodnih parov z moško neplodnostjo

Process of clinical diagnosis in the management of infertile couples with male infertility

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Dr. Andrea Garolla, dr. med., specialist farmakologije od leta 1999. Od leta 2001 je asistent v Centru za krioprezervacijo moških gamet. Njegova področja raziskovanja so: fiziopatologija spermalne in gonadne funkcije, neuroregulacija hipotalamo-hipofizno-gonadne osi, endokrini motilci in spermatogeneza, androgeni in metabolizem kosti, genetika primarnega hipogonadizma, kriokonzervacija gamet in rak testisa.

Povzetek

Pravilen diagnostični pristop k neplodnemu moškemu mora zajemati popolno splošno anamnezo, klinični pregled in laboratorijske analize, včasih pa vključuje tudi invazivno diagnostiko. Za zdravnika so odločilni pravilna določitev standardnih parametrov semena, mikrobiološka ocena in izključitev spermalnih protiteles. Pri neplodnih pacientih je pojavnost intraskrotalnih sprememb zelo visoka, zato je pomemben tudi pregled z Dopplerjevim ultrazvokom. Za popolno oceno hipotalamo-hipofizno-gonadne osi je potrebna osnovna hormonska ocena, ki zajema gonadotropine, totalni testosteron in estradiol. Tankoigelna aspiracijska citologija testisov je enostavna metoda za preučevanje spermatogeneze in je predvsem uporabna za razločevanje neobstruktivne od obstruktivne azoospermije. Fluorescentna in situ hibridizacijska analiza omogoča ugotavljanje anevploidije spermijev, še posebej je primerna v primerih ponavljajočih se splavov, kadar ima bolnik hujšo okvaro testikularnega tkiva ali je predhodno imel kemoradioterapijo. Prav tako obstaja kar nekaj testov, ki temeljijo na preučevanju funkcije spermijev, in bolje napovedujejo sposobnost oploditve, kot sta na primer ocena potenciala mitohondrijske membrane in določitev fragmentacije DNK spermijev. Zaključimo naj, da je klinična diagnoza neplodnega moškega proces, ki vključuje različne postopke. Samo popoln klinični in diagnostični pristop omogočata zdravniku, da izboljša zanositev po naravni poti ali s pomočjo tehnik asistirane reprodukcije.

Abstract

The correct diagnostic approach to an infertile male must provide a complete general history, physical examination and instrumental and laboratory analyses. A correct examination of standard sperm parameters, microbiological evaluation and exclusion of sperm antibodies are fundamental for the clinician. In infertile patients the incidence of intra-scrotal alterations is very high, therefore the Doppler ultrasound examination has a fundamental role. Basic hormonal evaluation of the infertile patient should include gonadotropins, total testosterone, prolactin and estradiol that allows the clinician to evaluate the whole function of the hypothalamo-pituitary-gonadal axis. Fine needle aspiration cytology of the testes is a simple option in the study of spermatogenesis, particularly useful to distinguish between secretory and obstructive azoospermia. Fluorescence in situ hybridization analysis allows the study of sperm aneuploidies, particularly suitable in cases of repeated abortions, in subjects with severe testicular damage and in subjects who previously had chemoradiotherapy. Finally, many tests based on the study of sperm function have been proposed to better predict fertility outcome such as the evaluation of mitochondrial membrane potential and the determination of sperm DNA fragmentation. In conclusion, the clinical diagnosis of the infertile patient is a process requiring many different competences. Only a complete clinical and diagnostic approach will allow the clinician to improve both natural and assisted fertility.

Ključne besede: Neplodni moški, anamneza, klinični pregled, pregled semena, mikrobiologija, ultrazvočni pregled modnika, hormoni, tankoigelna aspiracijska citologija testisov, anevploidije spermija, fragmentacija DNK, membranski mitohondrijski potencial, multidisciplinarnost.

Key words: Infertile male, anamnesis, examination, sperm analysis, microbiology, scrotum ultrasound, hormones, fine needle aspiration cytology of the testes, sperm aneuploidies, sperm DNA fragmentation, mitochondrial membrane potential, multidisciplinarity.

Medical history and physical examination

The key points of a correct diagnostic approach in medicine are represented by medical history and physical examination, therefore also the approach to the infertile patient must provide a complete and accurate general anamnesis and an objective examination. Moreover, instrumental checks and laboratory analyses will help the clinician to complete the diagnosis. Therefore, in the first phase the attention of clinician has to be specifically focused on the medical history (**Table 1**).

Table 1. *Scheme of complete medical and andrological history.*

Medical history data	-Age -Race -Religion -Job -Infertility -Infertility history	Urogenital surgery	Surgery for cryptorchidism -Orchiectomy -Herniorrhaphy -Funicular torsion Varicocelectomy Hydrocelectomy -Vasectomy -Epididimo-vasostomy -Vasostomy -Prostatectomy -Hypospadias -Circumcision
Familiar medical history	-Infertility -Spontaneous abortion -Born dead -Genetic and/or endocrine illnesses	Lifestyle and occupational medical history	-Exposure to occupational substances -Dietary habits -Sport -Alcohol consumption -Smoke -Illicit drugs -Steam bath -Tight pants
Pathological medical history	-High temperature in the previous months -Diabetes mellitus -Adrenal illnesses -Cystic fibrosis -Tuberculosis -Chronic infections -Allergy -Renal diseases -Hepatic diseases -Neurological diseases -Drugs	Sexual medical history	-Fertile period sexual intercourse -Sexual intercourse frequency -Libido -Erectile function -Ejaculate features -Orgasm features
Genital pathologies	-Cryptorchidism -Delayed or premature puberty -Testicular blow -Funicular torsion -Orchitis -Sexual transmission diseases -Epididymitis -Prostatitis -Vesiculitis -Urethritis -Genital dermatosis		

The evaluation of the infertile man cannot put aside from an accurate medical history on the type of working activity developed by the subject (contact with toxic or radioactive, repeated exposure to blows), and on possible pharmacological therapies, since many drugs interfere with the gonadal function as shown in **Table 2**.

Table 2. *Drugs potentially affecting andrological health.*

Hypogonadal hypogonadism	Hyperprolactinemia	Normogonadal or hypergonadal hypogonadism	Decrease of sperm movements	Obstructive pathologies
Androgens	Amitriptyline	Cytotoxic drugs	Atropine	Mercury
Cyproterone	Amphetamine	Antiinfectious drugs	Antidepressant drugs	
Medroxyprogesterone	Antidepressant drugs	Heroin	Antibiotics	
	Butyrophenone	Anesthetic drugs	Chlorpromazine	
	Estrogens	Barium	Diazepam	
	Imipramine	Cadmium	Local anesthetics	
	Methadone	Copper	Metoclopramide	
	Methyldopa	Mercury	Phentolamine	
	Metoclopramide	Pesticide	Propranolol	
	Morphine		Cadmium	
	Pimozide		Copper	
	Phenothiazine		Silver	
	Reserpine			
	Sulpiride			
	Tioxantine			

Physical exam will clarify the structural and morphological characteristics of external genitalia. Penile bendings, plates, inflammation, and warts should be excluded. Testicles should be evaluated to exclude hypotrophy, nodules, hydrocele and varicocele. Epididymis should be checked to point out enlargements and cysts. Presence of deferential ducts should be evaluated and finally volume and consistence of the prostate and seminal vesicles should be documented (**Table 3**).

Table 3. *Andrological examination.*

Physical examination	**Urogenital evaluation**
-Weight	-Penis
-Height	-Testis
-Blood pressure	-Epididymis
-General physical exam	-Vas deferens
-Secondary sexual characteristic	-Varicocele
-Gynecomastia	-Inguinal exploration
	-Rectal exploration

Sperm analysis

The standard examination of the sperm sample is a first-level analysis but it is fundamental to decide the following analyses and second-level exams. For a correct sperm analysis it is mandatory to standardize some rules of harvesting, delivery as well as of evaluation of the sperm sample. From many years, the manual of the WHO is considered the main reference at this aim. **Tables 4** and **5** report respectively the range for normal sperm parameters and their classification by the WHO manual.

Table 4. *Sperm parameters and normal values (WHO, 1999).*

Sperm parameters	**Normal limits**
Seminal volume	> 2 mL
pH	> 7.2
Sperm concentration/mL	> 20x 10^6
Total sperm count	> 40x 10^6
Motility (%)	> 50% progressive motility (grade a+b) o >25% rapidly progressive motility (grade a)
Morphology (%)	>30
Vitality (%)	>50
Leucocytes	< 1x 10^6 /mL
Immunobead test o MAR test	< 50% mobile spermatozoa with bead adhesion < 50% spermatozoa with particle adhesion

Table 5. *Classification of sperm parameters.*

Normozoospermia	Normal seminal parameters
Oligozoospermia	Sperm concentration < 20x 10^6/mL
Asthenozoospermia	< 50% spermatozoa with progressive motility (type A+B) or <25% spermatozoa with rapidly progressive motility (type A)
Teratozoospermia	< 30% spermatozoa with normal morphology
Oligo-astheno-teratozoospermia	Number, motility and morphology spermatozoa alterations.
Cryptozoospermia	Sperm cell recovery after centrifugation; spermatozoa absence in the ejaculate. (sperm concentration <1x 10^6/mL)
Azoospermia	No spermatozoa in the ejaculate also after centrifugation
Aspermia	Absence of ejaculate

Microbiological evaluation

The microbiological evaluation of the infertile man is based on the exclusion of possible infections of the urogenital tract that can be responsible for about 10-15% of all cases of infertility. Infections may affect the male genital tract at different sites, at the testis, epididymis and sexual accessory glands. In the case of testicular and/or epididymal infection, sperm can be impaired at different phases of their development and maturation, with consequent alteration of spermatogenesis leading to quantitative and/or qualitative worsening of sperm parameters. Furthermore, some infections may contribute to infertility through different mechanisms: production of cytokines as interleukin 1 and interleukin 8 that are toxic for sperm cells, reduction of sperm motility or alteration of the acrosome reaction due to bacteria such as Mycoplasma and Chlamydia trachomatis. Moreover, alterations of the biological composition and biochemistry of the seminal plasma may lead to a significant reduction of the fertilizing potential of sperm cells. Finally, the inflammation due to infection, besides the alteration of the structure of the pelvic organs, can provoke dysfunction of the pelvic floor and obstruction of the male genital tract. Recently, some studies have demonstrated that HPV can be present in sperm cells of adult males who had had unprotected intercourse. In addition, HPV DNA localizes in the head of sperm and even it is still unclear whether it integrates into the nucleus or not, its presence seems to be frequently associated with reduced sperm motility. Therefore, further studies are needed to clarify these findings and to investigate a possible role of HPV in a larger number of men affected by asthenozoospermia or as partners of women reporting precocious recurrent miscarriage. HPV persistence could also be involved in the impairment of sperm parameters, in particular sperm motility, and suggests caution in the use of these cells for assisted reproduction techniques or sperm banking.

Ultrasound examination

In infertile patients the incidence of scrotal alteration is very high with a prevalence ranging from 59% up to 72%. Therefore it is clear that the Doppler ultrasound examination has a fundamental role in the study of alterations of the male genital tract leading to infertility. These abnormalities are represented by the following: hydrocele, varicocele, testicular and/or epididymal lesions, testicular hypotrophy, testicular microlithiasis and so on. Furthermore, in some cases the ultrasonographic evaluation of the prostate gland and seminal vesicles is mandatory, particularly when infection, inflammation and asthenoteratozoospermia with normal sperm count occur. Also in cases of ejaculatory disorders (early, late, painful and absent) the ultrasound evaluation of accessory glands should be performed.

Hormones

The basic hormonal evaluation of the infertile patient should include gonadotropins (LH and FSH), total testosterone (T), prolactin, and estradiol. In the evaluation of T plasma levels, it is important to keep in mind that this hormone has a circadian secretion with maximum plasma values in the early morning and minimal during the evening. The contemporary dosage of hormones allows the clinician to evaluate gonadal function and in case

of dysfunction it may suggest the site of hypothalamo-pituitary-gonadal axis alteration (**Table 6**).

Table 6. *Hormonal pattern and infertility causes.*

	LH	FSH	T	
1	N	N	N	Post-testicular causes, testicular causes (unilateral or bilateral diseases)
2	↑	↑	↓	Testicular causes (bilateral diseases)
3	N	↑	N	Testicular causes (tubular component involvement)
4	↓	↓	↓	Pre-testicular causes
5	↓	↓	↑	Pre-testicular causes (androgens administration, solid tumors secreting androgens)
6	↑	↑	↑	Testicular causes (AIS)

The whole study of the hypothalamo-pituitary-gonadal axis could be useful to complete the andrological diagnosis. In presence of signs and symptoms of adrenal hyper- or hypofunction (hypertension, obesity, stripes or hypotension, weight decrease, asthenia, melanodermia) the evaluation of the urinary free cortisol and the plasmatic cortisol should be performed. The determination of steroidogenesis metabolites (17-OHprogesterone, progesterone, pregnenolone, androstenedione), of T and dihydrotestosterone both basal and after hCG administration, can be suitable to study congenital defects of steroidogenesis and to evaluate the androgenic function (**Table**7).

Table 7. *Hormones and clinical significance.*

Hormones	**Time of evaluation**	**Normal range**	**Clinical significance**
Total testosterone	8:00	2.8-11 ng/mL	It indicates the secretory activity of Leydig cells. Low levels of T associated with low levels of gonadotropins are indicative of hypogonadotropic hypogonadism. Low levels of T associated with high levels of gonadotropin are indicative of primitive or hypergonadotropic hypogonadism.
Free-testosterone	8:00	56-200 pg/mL	A normal men has a 2% of free testosterone plasmatic levels. Free testosterone is useful in subjects with alterations of SHBG levels (obesity, hyperthyroidism, hepatic cirrhosis).
SHBG		0.5-1.5 nmol/L	It is high in old and hyperthyroid subjects. It is low in obesity.
FSH	8:00	1.5-11 UI/L	It indicates the levels of spermatogenetic function. High levels indicate alteration of tubular germinal cells and are useful as negative prognostic factor for testicular spermatozoa retrieval.
LH	8:00	1.5-9 UI/L	It stimulates testosterone production by Leydig cells.

Hormones	Time of evaluation	Normal range	Clinical significance
Estradiol	8:00	10-60 pg/mL	It is produced by Leydig cells or testosterone peripheral aromatization. It is high in cases of increased aromatase activity (obesity) or testis primary hyperproduction (testicular secreting neoplasia).
βhCG		<5 mUI/mL	It is high in testicular germinal neoplasia.
Prolactin	8:00	4-11 ng/mL	The dosage is useful in case of low levels of T and high levels of LH. Sometimes it is high in renal failure, cirrhosis, hypothyroidism, drugs assumption (es. Antidopaminergic and serotoninergic drugs, proton pump inhibitors, verapamil), hypothalamic and pituitary tumors.
DHT		25-75 ng/mL	It is low in case of 5-alpha-reductase deficiency.

Testicular fine needle aspiration and cytology

Fine needle aspiration cytology (FNAC) of the testes was proposed in 1992 and has been demonstrated to represent a minimally invasive and reliable parameter in the study of the seminiferous epithelium and spermatogenic process in severely infertile men. The mean value of each cell type observed in normal spermatogenic process is shown in **Table 8**.

Table 8. *Mean value of each cell type in normal spermatogenesis.*

Cellular type	Mean ± DS
Spermatogonial	2.5 ± 2.2
Primary spermatocyte	6.0 ± 3.6
Secondary spermatocyte	3.9 ± 2.3
Spermatid Sab	15.2 ± 5.8
Spermatid Scd	37.9 ± 12.9
Spermatic Index (SI)	34.8 ± 13.3
Sertolian Index (SEI)	30 ± 19.5

Testicular cytological analysis is relatively simple and allows the identification of different clinical groups of infertile subjects and different kinds of testicular tubular alteration. This exam is particularly useful in cases of numeric alteration of spermatozoa (especially azoospermia and cryptozoospermia) to distinguish between secretory and obstructive form and to evaluate the spermatogenetic line into the testis in cases of maturation arrest (**Table 9**).

Table 9. *Interpretation of fine needle aspiration cytology results.*

Seminal	Cytology	Fine-needle results	Hormones
Azoospermia	-Obstruction. -Sertoli cell-only syndrome -Hypospermatogenesis -Complete maturation arrest at spermatogonial/spermatocytic or spermatidic level	-Normal germinal maturation line -Germinal elements completely absent -Reduction of the germinal component SEI index high (>300); absence of mature spermatic cells -Normal SEI index, completely absence of mature cells and increase of spermatogenetic cells above the arrest (spermatogonial/spermatocytes or spermatocytes/spermatids)	-Normal FSH -High FSH -High FSH -High FSH in precocious arrest; normal in delayed maturation arrest (normal hypophysis feed-back)
Oligozoospermia	-Moderate hypospermatogenesis -Partial maturation arrest at spermatogonia/ spermatocytes or spermatid level	-Increase of SEI index (50-300); reduction of germinative component and relative increase of Sertoli cells; decrease of mature spermatic cells - Normal SEI index, decrease of mature cells and increase of spermatogenetic cells above the arrest	-High FSH - High FSH in precocious arrest; normal in delayed maturation arrest (normal hypophysis feed-back)

Furthermore, FNAC can be used as a prognostic tool of sperm recovery by TESE in non-obstructive azoospermia and to retrieve sperm for assisted reproductive procedures in obstructive azoospermia. Finally, this procedure is fundamental to choose the medical treatment in cases of severe oligozoospermia.

Sperm multicolour fluorescence *in situ* hybridization analysis

The multicolour FISH (fluorescence in situ hybridization) analysis allows the study of sperm chromosomes and to evaluate the percentage of sperm aneuploidies. Such technique is able to locate specific sequences of nucleic acid in the sperm head, through the use of multicolour fluorescent complementary nucleotide sequences, named probes. This procedure is particularly suitable in cases of repeated reproductive failures, but its applications are manifold: as prenatal test, as preimplantation analysis to foretell the result of pregnancy and as postnatal test to evaluate chromosomal abnormalities of the baby. This test is mandatory to evaluate sperm aneuploidies in those subjects who previously had chemoradiotherapy for previous cancer, before seeking fertility.

Furthermore, the study of sperm numerical chromosomal aberrations seems to be particularly important in those subjects with severe testicular damage. In fact, infertile subjects frequently show increased percentages of sperm alterations and, in particular, spermatozoa of patients with severe testicular damage have been shown to contain nuclear alterations including abnormal chromatin structure, DNA strand breaks and aneuploidies. Moreover, the increased aneuploidy rate frequently reported in these subjects raises important concerns regarding the potential of intracytoplasmic sperm injection to facilitate the transmission of genetic diseases. Finally, because the detection of HPV in the sperm head is easy to

achieve with FISH analysis, we suggest using this technique as a first-step screening for men with risk factors for HPV infection, before performing assisted reproduction techniques or sperm banking.

Tests of sperm function

Male infertility is commonly defined in relationship to the conventional sperm analysis, that gives information about the concentration and number of sperm in the ejaculate, the percentage of sperms with normal viability, motility and morphology. However, it is well known that the relation between these conventional criteria and the fertility potential is not direct and in many cases these parameters are unable to explain the causes of infertility. Sperm fertilizing potential, embryo development and early pregnancy loss depend on the integrity and function of different sperm structures and in particular DNA. To this aim in the last years many tests based on the study of these structures have been proposed to better predict fertility outcome (**Table 10**). An example of such a test is the evaluation of mitochondrial membrane potential, whose alteration seems to be suggestive of an early apoptotic process and is frequently observed in sperm samples from infertile men. Again, the determination of sperm DNA damage (i.e. DNA fragmentation and abnormal DNA integrity) is strongly recommended when advanced forms of assisted reproductive techniques are used.

Table 10. *Tests of sperm function and their clinical significance.*

Tests of sperm function		
Test	**Alteration**	**Clinical significance**
Annexin V	Phosphatidylserine externalization	Initial apoptosis
JC-1	Mitochondrial membrane potential	Initial apoptosis
Acridine orange	Chromatin integrity	Alteration of DNA double strand, late apoptosis
TUNEL	Nuclear DNA fragmentation	DNA fragmentation, late apoptosis
Aniline	Nuclear DNA protamination	Nuclear maturation process alteration
Nuclear condensation	Nuclear condensation	Altered protamination
FISH	Sperm aneuploidies	Sperm chromosome number alterations

Conclusions

In conclusion, the clinical diagnosis of the infertile patient is a complex process requiring many different competences: general medicine, endocrinology, physiology, urology, cell biology, cytology, microbiology, immunology, ultrasound and molecular biology. Only a so wide approach to andrological problems will allow the clinician to perform a correct diagnosis and to improve sperm parameters. Even if the improvement of these parameters infrequently leads to reach spontaneous pregnancy, it is a fundamental step to increase chances of fertility by assisted reproduction tools.

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Diagnostika in zdravljenje azoospermije in aspermije

Azoospermia and aspermia: diagnosis and treatment

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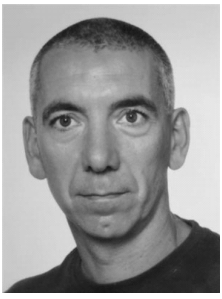


Sašo Drobnič je specialist ginekologije in porodništva (Ljubljana, 1992).

Od leta 1987 je v službi na Ginekološki kliniki v Ljubljani na oddelku za reprodukcijo. Ukvarja se z zdravljenjem neplodnih parov, moške in ženske neplodnosti in sodeluje v raziskavah na omenjenih področjih.

Povzetek

Azoospermija in aspermija sta skrajni obliki moške neplodnosti. Za azoospermijo je značilna odsotnost semenčic v semenskem izlivu. Delimo jo na neobstruktivno in obstruktivno. Obstruktivno azoospermijo (OA) povzroča motnja v prenosu semenčic iz mod v izliv skozi nadmodek, semenovod in sečnico. OA je lahko prirojena, v razmeroma redkih primerih prirojene agenezije obeh semenovodov (CBAVD), ali pridobljena, največkrat zaradi posledic vnetja pomožnih žlez ali zaradi kirurškega posega (sterilizacija, operacija kile). Neobstruktivno azoospermijo (NOA) pa povzroča nezmožnost mod ustvarjati semenčice zaradi pomanjkljive hormonske spodbude s strani sistema hipotalamus-hipofiza (pretestikularni vzrok) ali pa je vzrok zanjo v tkivu testisa samem. Nekatere OA lahko zdravimo kirurško z epididimovazostomijo, primere CBAVD in NOA pa z biopsijo testisov, izolacijo spermija in s postopkom ICSI (TESE-ICSI). Posebej pomemben je predvsem v primeru NOA tudi histološki pregled tkiva, pri katerem iščemo displazije.

Aspermija je odsotnost izliva zaradi anejakulacije ali retrogradne ejakulacije. V večini primerov jo povzroči poškodba hrbtenjače, lahko pa so vzroki iatrogeni (kirurški poseg) ali degenerativni (multipla skleroza, sladkorna bolezen). Za retrogradno ejakulacijo je značilna prisotnost semenčic v urinu po samozadovoljevanju. Po alkalinizaciji urina lahko semenčice uporabimo za oploditev v postopkih ICSI. Pri ostalih je možnost zdravljenja z ICSI odvisna od prisotnosti semenčic v tkivu testisa po biopsiji. V primerih azoospermije in aspermije pri partnerkah naredimo le osnovne preiskave plodnosti (ultrazvok, določanje hormonov).

Abstract

Azoospermia and aspermia are extreme forms of male infertility. Azoospermia i.e. absence of spermatozoa in the ejaculate can be divided into obstructive and nonobstructive. Obstructive azoospermia (OA) is caused by impaired transfer of sperm into ejaculate via epididymis, ductus deferens and urethra (post testicular form). OA can be congenital, as in the case of bilateral absence of the vas deferens (CBAVD), or acquired, mostly due to accessory gland inflammation, but also after trauma or surgery (vasectomy, herniorrhaphy). Non obstructive azoospermias (NOA) are characterized by impaired spermatogenesis due to pretesticular (insufficient hormonal stimulation by the hypothalamo-hypophyseal system) or testicular causes. While some OA can be treated surgically by performing epididymovasostomy, NOA and CBAVD are treated by testicular biopsy, sperm extraction and ICSI (TESE-ICSI). Particularly in cases of NOA, histological examination of testicular tissue must be performed and signs of dysplasias looked for.

Aspermia is the inability to ejaculate due to anejaculation or retrograde ejaculation. In most cases, aspermia is caused by spine injury. Other causes for aspermia are iatrogenic (surgical) and degenerative (diabetes, sclerosis multiplex). In cases of retrograde ejaculation the diagnosis is made upon the presence of sperm in the urine after masturbation. After urine alkalinization spermatozoa can be used for assisted reproductive techniques. In other cases the chances for successful procedure depend on the presence of spermatozoa in the testicular tissue after biopsy. In cases of azoospermia and aspermia, minimal diagnostic management (ultrasound examination, hormone assessment) of the female partner is required.

Ključne besede: Azoospermija, aspermija, biopsija testisa, epididimovazostomija, genetski vzroki, TESE-ICSI.

Key words: Azoospermia, aspermia, testicular biopsy, epididymovasostomy, genetic causes, TESE-ICSI.

Uvod

Azoospermija je stanje, pri katerem semenčic v bolnikovem semenskem izlivu ne najdemo. Do nje pride, kadar se semenčice v modih ne tvorijo (neobstruktivna azoospermija, NOA) ali kadar je prenos semenčic v izliv preprečen (obstruktivna azoospermija, OA).

Odsotnost izliva semena imenujemo aspermija. Povzroči jo bodisi popolna odsotnost izliva (anejakulacija) ali pa je izliv nasproten (retrogradna ejakulacija). Vzroki za aspermijo so praviloma nevrološke okvare, redko so psihološki.

Z uvedbo biopsije mod, izolacije in vnosa spermija v jajčno celico (TESE-ICSI) v terapijo azoospermije in aspermije leta 1994, se je obravnava le-teh popolnoma spremenila. Pri zdravljenju aspermije in azoospermije zdaj opuščamo, razen najnujnejših (ultrazvok rodil, analize koncentracij hormonov v serumu), ostale preiskave partnerk, ker jih z njimi le nepotrebno obremenjujemo.

Azoospermija

Definicija, incidenca

Za azoospermijo je značilna popolna odsotnost semenčic v semenskem izlivu v vsaj dveh vzorcih, ki jih pregledamo v intervalu vsaj enega meseca. Izliv pregledujemo po navodilih Svetovne zdravstvene organizacije (WHO, 1999): vzorec centrifugiramo pri 3000 *g* 15 minut, nato pod mikroskopom pregledamo usedlino.

Vzroki azoospermije so motena, odsotna ali nezadostna proizvodnja semenčic v modih (NOA), ali preprečen prenos semenčic v izliv (OA). Pogostnost azoospermije v splošni populaciji je 1,6%, med neplodnimi pari 6%, med neplodnimi moškimi pa 10-15%.

Razvrstitev

NOA najpogosteje povzroči nezmožnost mod, da bi normalno sintetizirala semenčice. Tako stanje je lahko prirojeno ali pridobljeno. Med azoospermijami je neobstruktivnih približno 80% . Če upoštevamo histološki izvid tkiva mod, jih lahko razvrstimo v več vzročnih skupin:

- Hipospermatogeneza
- Sindrom Sertolijevih celic
- Zastoj v dozorevanju
- Skleroza semenskih kanalčkov

OA je lahko prirojena ali pridobljena. V večini primerov je nastajanje semenčic v modih nemoteno, semenčic v izlivu pa vseeno ni. Vzroke obstruktivne azoospermije lahko delimo na:

- Obstrukcijo nadmodka.
    - - Prirojena
    - - Pridobljena po vnetju ali kirurškem posegu
- Obstrukcijo semenovoda
    - - Prirojena (agenezija obeh semenovodov-CBAVD)
    - - Pridobljena: po kirurškem posegu (vazektomiji, operaciji kile, drugih posegih na mošnji).
- Prirojena ali pridobljena obstrukcija ejakulatornih vodov.

Preiskave

Pri ločevanju med NOA in OA nam pomagajo klinični pregled, merjenje hormona FSH, histološki pregled mod in določanje prisotnosti semenčic v tkivu mod (Rowe *et al*., 2000).

Pri anamnezi pacienta povprašamo po družinskih značilnostih npr. degenerativnih boleznih pri prednikih in zlasti po neplodnostih pri očetu ali bratih. Preverimo zgodovino njegovih bolezni, zlasti degenerativnih, malignih in vnetnih bolezni spolovil ter način njihovega zdravljenja (npr. obsevanje, kemoterapija, zlasti z alkilirajočimi agensi in druga zdravila, ki znano okvarjajo nastajanje semenčic v modih), preverimo zgodovino posegov na spolovilih

(npr. operacije kriptorhizma, varikokele). Bolnika vprašamo po navadah (rednost in ustreznost spolnih odnosov) in razvadah (kajenje, prekomerno pitje alkoholnih pijač in uživanje mamil).

Splošna preiskava obsega oceno bolnikove postave: rast, obseg in porazdelitev mišične mase, vzorec poraščenosti, prisotnost ginekomastije in znakov drugih bolezni.

Pri preiskavi bolnikov z NOA običajno najdemo testise manjše velikosti. Pri nekaterih enega ali obeh testisov v mošnji ne najdemo (enostranski ali obojestranski kriptorhizem), najdemo lahko sledi operativnega spuščanja mod v mošnjo (orhidopeksije). Pogostejše so nepravilnosti penisa (hipospadija) ali pa je le-ta manjši. V mošnji iščemo sledi prisotnosti kile ali razširjenih žil, tudi ob pritisku (manever po Valsalvi).

Najpomembnejša preiskava bolnikov z azoospermijo je določanje koncentracije folikle stimulirajočega hormona (FSH) v serumu. Za večino bolnikov z NOA je značilna povišana koncentracija FSH v serumu. Redkeje je koncentracija FSH prenizka. Na podlagi prenizke koncentracije FSH in nekaterih anamnestičnih podatkov (kasna puberteta po nadomestni terapiji) sklepamo na t.i. hipogonadotropni hipogonadizem, stanje, ki ga povzroči nezadostno spodbujanje delovanja mod s strani hipotalamično-hipofiznega sistema (Kallmannov sindrom).

Bolnika pošljemo na genetski pregled, kadar posumimo na dedni vzrok neplodnosti. Pri bolnikih z značilno postavo (široki boki, ginekomastija, dolgi udi, majhna moda) s preiskavo kariotipa lahko potrdimo prisotnost dodatnega kromosoma (47XXY, Klinefelterjev sindrom). Pri bolnikih z neobstruktivno azoospermijo v 5-10% najdemo mikrodelecijo na dolgem kraku kromosoma Y (regije AZF). Drugi genetski vzroki azoospermije so redkejši.

Ultrazvočni pregled mod naredimo sistematično pri moških z anamnezo retiniranega moda, pri tipnih spremembah na modih ali kadar sumimo na obstrukcijo ejakulatornih vodov.

Pridobljena azoospermija je v večini primerov posledica zdravljenja. Kemoterapija, zlasti uporaba alkilizirajočih zdravil lahko nepovratno okvari spermatogenezo. Koncentracija serumskega FSH je pri takih bolnikih povišana, občasno najdemo tudi druge znake okrnjene funkcije mod, predvsem pri tistih, ki so se zdravili v rani mladosti.

Vnetje mod (orhitis), poškodba ali torzija povzročijo azoospermijo le takrat, kadar so obojestranske in jih spremlja atrofija mod. Zloraba anabolikov pri mladih težkoatletih pa lahko povzroči azoospermijo zaradi vpliva visoke koncentracije testosterona na koncentracijo FSH v serumu.

Večino OA povzroči vnetje pomožnih spolnih žlez. Nanjo posumimo, kadar poleg anamnestičnih podatkov o prebolelem vnetju ob pregledu najdemo povečane, včasih občutljive nadmodke ob sicer normalno velikih modih. Koncentracija FSH je v takih primerih normalna.

Redkeje OA povzroči prirojena odsotnost semenovodov, stanje, ki ga v večini primerov povzroči mutacija gena CFTR. Moda so običajno primerne velikosti, nastajanje semenčic je neokrnjeno, semenovodi netipljivi, koncentracija FSH v serumu pa normalna.

Zdravljenje

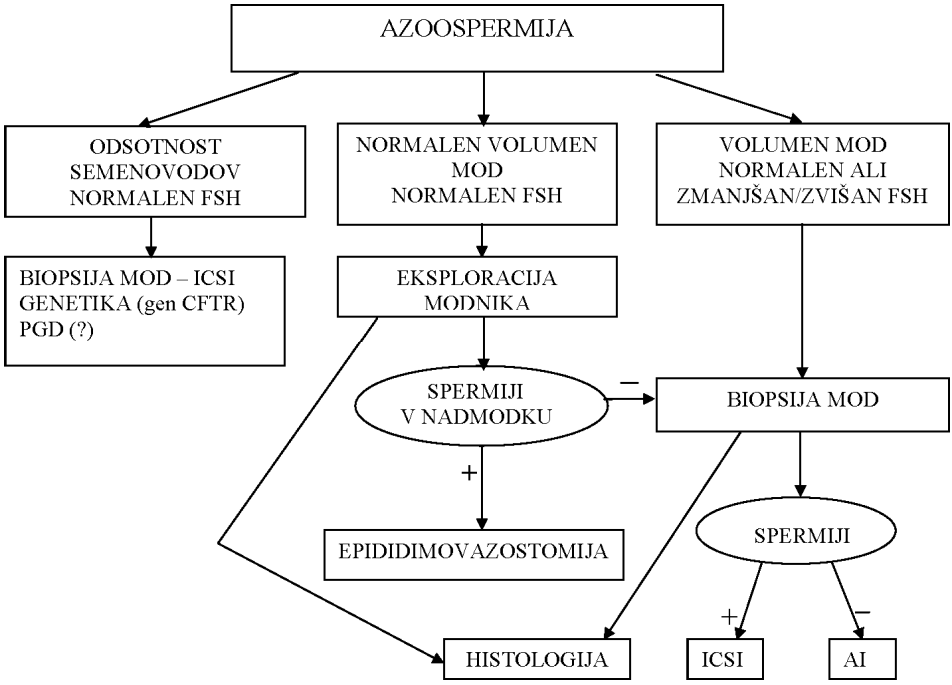
Nekatere bolnike z OA (<10% vseh bolnikov z azoospermijo) lahko zdravimo kirurško. Zapore po vnetjih so najpogostejše v repu nadmodka ali ob stiku nadmodka in semenovoda. Če ob vrezu nadmodka med operacijo in pregledu dobljene tekočine iz njegove svetline najdemo številne semenčice, naredimo mikrokirurško povezavo med nadmodkom in zdravim delom semenovoda, epididimovazostomijo. Med posegom vedno naredimo tudi biopsijo testisa. Dobljeno tkivo pregledamo, če so v njem semenčice, tkivo zamrznemo, manjši del pa pošljemo v histološki pregled. Z dobljenimi semenčicami lahko oplodimo jajčne celice v postopku zunajtelesne oploditve in prenosa zarodka (TESE-ICSI). Po zdravljenju z epididimovazostomijo tretjina žensk spontano zanosi, v ejakulatu dveh tretjin moških pa so po posegu prisotni spermiji (Smrkolj *et al.*, 2009).

Bolnikom s prirojeno obojestransko agenezijo semenovodov prav tako naredimo biopsijo testisov za postopek ICSI. Obema partnerjema predlagamo tudi genetski pregled in v primeru mutacije gena CFTR pri obeh, paru ponudimo predimplantacijsko genetsko preiskavo (PGD), da preprečimo prenos cistične fibroze na potomce.

Bolnike z NOA in nizko koncentracijo FSH in LH (hipogonadotropni hipogonadizem, Kallmannov sindrom) zdravimo z odmerki HCG, kasneje pa s kombinacijo HCG in HMG. Ko se med zdravljenjem v izlivu pojavijo semenčice jih zamrznemo za postopek ICSI. Bolnikom, pri katerih kljub zdravljenju semenčic v izlivu ne najdemo, naredimo biopsijo testisa in zamrznemo tkivo s semenčicami (Zorn *et al.*, 2005). Zdravljenje s testosteronom, ki ga bolniki sicer prejemajo med nadomestnim hormonskim zdravljenjem, ukinemo zaradi njegovega škodljivega vpliva na nastajanje semenčic v modih.

Bolnikom z NOA in povišano koncentracijo FSH v serumu naredimo biopsijo testisov v lokalni anesteziji. V dobljenem tkivu iščemo semenčice in ga, če so v njem semenčice (v približno polovici vseh primerov), zamrznemo za postopek TESE-ICSI. Tkivo pregledamo tudi histološko, opredelimo motnjo spermatogeneze (točkovanje po Johnsenu) in pri bolnikih z visokim tveganjem za karcinom moda iščemo spremembe, značilne za karcinom *in situ*. Če v tkivu semenčic ni, partnerjema predlagamo darovalski postopek ali posvojitev (Slika 1)

Slika. 1. Diagnostika in zdravljenje azoospermije.



Aspermija

Diagnoza in zdravljenje

Odsotnost izliva semena imenujemo aspermija. Povzroči jo bodisi popolna odsotnost izliva (anejakulacija) ali pa je izliv nasproten (retrogradna ejakulacija). Najpogostejši vzrok aspermije je poškodba hrbtenjače nad segmentom T 10. Drugi vzroki zanjo so poškodbe medenice, odstranitev retroperitonealnih bezgavk med terapijo tumorjev testisa, multipla skleroza in diabetična nevropatija. Diagnozo retrogradne ejakulacije postavimo, kadar najdemo semenčice v urinu po masturbaciji. V takih primerih urin alkaliniziramo z nekaj odmerki sode bikarbone dan pred odvzemom, gibljive semenčice iz urina pa zamrznemo za postopek ICSI. Pri ostalih je možnost zdravljenja odvisna od prisotnosti semenčic v tkivu testisa po biopsiji (Zorn *et al.*, 2006).

Stimulacija ejakulacije z -simpatikomimetiki, neostigminom in elektroejakulacijo se zaradi možnih resnih zapletov (hipertenzivna kriza, avtonomna disrefleksija) marsikje opušča.

Primere psihogene anejakulacije lahko uspešno zdravimo s stimulacijo ejakulacije z vibracijo glavice penisa, dobljeno seme pa uporabimo v postopkih osemenitve (IUI) ali IVF-ET.

Postopek OBMP pri NOA, OA in aspermiji z uporabo semenčic iz moda oz. nadmodka

Naše izkušnje (Zorn et al., 2009) kažejo, da je stopnja zanositve po ICSI-TESE primerljiva s tisto, doseženo s semenčicami iz ejakulata. Več blastocist se razvije ob uporabi zamrznjenih in gibljivih spermijev in več otrok se rodi ženskam, mlajšim od 38 let.

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Klasična analiza semena in poškodbe kromatina spermija

Classical semen analysis and sperm chromatin damage

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Avtor je član komiteja za peto izdajo Priročnika Svetovne zdravstvene organizacije za analizo semena. Je predsednik Skandinavskega združenja za andrologijo (NAFA) in koordinator Tečaja osnovne analize semena. Sodeluje pri Programu zunanjega vrednotenja kakovosti za andrologijo v združenju ESHRE.

Povzetek

Priročnik Svetovne zdravstvene organizacije za analizo semena je osnova za delovanje laboratorijev. Priporoča metode, s katerimi pridobimo najbolj zanesljive podatke. Pri vrednotenju analize semena je najpomembnejše prepoznavanje dejavnikov, ki lahko spremenijo končne rezultate analize, ne da bi bila dejansko prisotna bolezen ali motnja. Z analizo semena dobimo pomembno informacijo o moških reproduktivnih organih, medtem ko je napovedna vrednost posameznih parametrov semenske analize za izid zdravljenja omejena. S klasično analizo semena pridobimo podatke o količini in kakovosti količini in kakovosti nastajanju semena in o prenosu spermijev ter podatke o osnovni funkcionalni sposobnosti spermijev, sposobnih gibanja. Nobena od teh kvalitet ni v neposredni povezavi s stanjem genetskega materiala v glavi spermija. Verjetno obstaja negativna povezava med označevalci poškodb na DNK spermijev in izidom nosečnosti. Za klinično rabo mejne vrednosti še niso določene, najverjetneje zaradi tega, ker »DNK testi spermijev« ne določajo samo že obstoječih napak na DNK. Odvisni so od dostopnosti do DNK, kar pa je odvisno tudi of organiziranosti kromatina. Poleg sprememb v strukturi kromatina med formiranjem in prenosom spermijev po moškem reproduktivnem organu nastajajo še hitre a individualno zelo nihajoče spremembe v kromatinu, kadar je le-ta izpostavljen laboratorijskemu okolju, kot je npr. povečan oksidativen stres ob nefiziološki izpostavljenosti semenske tekočine.

Abstract

The WHO manual on semen analysis is a handbook for the laboratories providing advice on methods most likely to deliver robust and reliable data. For the interpretation of semen analysis results it is, however, essential to recognize a number of factors that can alter results without representing real disorders or diseases. Semen analysis can give valuable information about the male reproductive organs, while the prognostic information for the fertility outcome of the couple is limited when semen characteristics are evaluated separately from each other. Classical semen analysis concerns the quantitative and qualitative production and transport of sperm, along with the basic functional capacity of sperm to move forwards. None of these qualities is directly related to the status of the genetic material in the sperm head and there appears to be an inverse relation between markers for sperm DNA damage and fertility outcome. However, for clinical use no clear cut-off limits have been identified. This is likely to be due to that the "sperm DNA tests" do not only test already existing DNA damage: they are dependent on the access to the DNA, and this access is also dependent how the chromatin is organized. Besides the changes in sperm chromatin structure during sperm formation and transport through the male reproductive organs, rapid but individually very varying changes occur in the sperm chromatin when exposed to the laboratory environment, for instance non physiological exposure to seminal vesicular fluid causing increased oxidative stress.

Ključne besede: Klasična analiza semena, oploditev, fiziološke in patološke spremembe DNK, nastajanje in prenos spermijev, oksidativni stres, priročnik Svetovne zdravstvene organizacije.

Key words: Classical semen analysis, fertility, physiological and pathological DNA changes, sperm production and transport, oxidative stress, WHO manual.

Introduction

The call for standardized and controlled methods for the examination of human ejaculate can be traced for more than 60 years back (Harvey and Jackson, 1945). In the 1950ies, the legendary John MacLeod published studies of reproductive physiology, semen analysis and fertility outcome (MacLeod and Gold, 1951). Further development of methods appeared as consistent laboratory science was applied in the field (Eliasson, 1975, Mortimer, 1985), followed by the development of systematic training programs (Mortimer, 1994a, Mortimer, 1994b, Björndahl *et al.*, 2002). More recently the implementation of schemes for external quality control (Matson, 1995, Libeer *et al.*, 1996, Cooper *et al.*, 1999, Cooper *et a*l., 2002, Alvarez *et al.*, 2005) has provided further support for global improvements of modern laboratory andrology. WHO guidelines from 1980 and onwards (World Health Organization, 1999) have been most important for these achievements. Originally, the guidelines were not detailed enough for practical work in laboratories. The Nordic Association for Andrology (NAFA) developed a basic laboratory handbook. This "cookbook" was adapted to the 4th WHO manual in collaboration with the Special Interest Group in Andrology (SIGA) of the European Society of Human Reproduction and Embryology (ESHRE) (Kvist and Björndahl, 2002). In conclusion, there is easily available information

about reliable and efficient methods, systematic training for these methods, and quality control schemes to maintain a high level of quality performance. Surprisingly, many laboratories still do not apply these methods, implement systematic training and run internal and external quality control (Keel *et al.*, 2002, Riddell *et al.*, 2005)!

In recent years the interest in the sperm DNA has increased dramatically. While the classical semen analysis deals with the production and transport of the *messenger* - the courier - sperm DNA assessments are assumed to give information about disturbances of the *message*.

The focus of this presentation is what help basic semen analysis can provide and what further information can be obtained from sperm DNA tests, by discussing the limitations of each method.

Basic Semen Analysis

The presence of spermatozoa in the ejaculate is a basic requirement for *in vivo* fertility, and can be done with a high degree of certainty by examination under a phase microscope. Still, concentration is dependent on the dilution of spermatozoa by prostatic and seminal vesicular fluids, and the total number of spermatozoa is also dependent on the recruitment of spermatozoa from sperm storages in the epididymis (Pound *et al.*, 2002). Therefore, variation in sperm numbers and concentration observed among different ejaculates is not only due to diseases or disorders.

Among qualitative aspects the visual appearance of fixed, stained and mounted smears of spermatozoa under a high power microscope (i.e. sperm morphology) has been shown to be an important marker for decreased success in vivo and in vitro (MacLeod and Gold, 1951, Jouannet *et al.*, 1988, Menkveld *et al.*, 1990). Decreased proportion of spermatozoa with morphology typical of spermatozoa able to reach the site of fertilization does indicate a relatively reduced probability for pregnancies (Menkveld *et al.*, 1990).

The absence of rapid progressively moving sperm is a strong negative factor for the success of IVF (Verheyen *et al.*, 1999, Sifer *et al.*, 2005). The causes for decreased sperm motility could be infections with inflammatory reactions, but also a decreased secretory function of the prostatic gland. Therefore, it is useful to measure the markers for contributors to the semen volume (zinc: prostate; fructose: seminal vesicles; -glucosidase: epididymis). In some men the presence of antisperm antibodies can cause reduced or total lack of motility. If most spermatozoa are immotile a vitality test is indicated to determine if immotile spermatozoa are dead or alive and immotile. Reduced sperm motility can in some men be due to abnormal sequence of ejaculation where most spermatozoa are expelled together with seminal vesicular fluid (instead of the prostatic fluid). A possible cause for this disorder could be ejaculatory duct obstruction, delaying the emptying of the spermatozoa into the urethra (Fisch *et al.*, 2006). Abnormal sequence of ejaculation can only be discovered by examining split ejaculate fractions (Björndahl and Kvist, 2003).

A vast majority of all queries regarding the 5th edition of the WHO manual have been related to the reference ranges. The data used for establishing a reference range come from recent fathers. This means that if the semen analysis results of a man in an infertile couple

are outside the 95% limits of recent fathers, only 5% of recent fathers have as extreme values, but it says nothing about what is common among men in infertile couples. We would need also the distribution of results from truly infertile men in order to make valid conclusions.

A more successful way to use semen analysis results for prognostic information is to look at more than one parameter at the same time (Guzick *et al.*, 2001, Jedrzejczak *et al.*, 2008). Furthermore, semen analysis should not be interpreted without the information about the patient history, physical examination and other laboratory investigations (Jequier, 2006).

Sperm DNA Tests

The organization of the sperm nucleus is completely different compared to somatic cells, but still the test such as the TUNEL or the Comet assay are adaptations from the tests for DNA damages in somatic cells. Most of DNA in the sperm head is inactivated and densely packed. The structural organization hinders access to the sperm DNA and protects the DNA from damage caused by radiation, temperature and different water soluble chemicals. Due to a very strong structure, all protocols for sperm DNA staining must include steps to break the sperm specific chromatin structure. In short, the tests appear to be able to reveal how easy it is to access the DNA; it has not been proven that the tests actually reveal already existing damages to the DNA. However, also increased access to the DNA could indicate a problem: DNA with high degree of access is more likely to be damaged.

At ejaculation the sperm chromatin has a rapidly reversible chromatin stability that depends on the presence of zinc within chromatin. When spermatozoa are stored in vitro, this zinc-stability is superseded by disulfide-bridge stability (Björndahl and Kvist, 1985). This change is enhanced if zinc is extracted from chromatin (e.g. by seminal vesicular fluid in semen) (Kvist and Björndahl, 1985). Increased disulfide-bridge stabilization is likely to delay the normal chromatin decondensation within the ooplasm after fertilization which could contribute to post fertilization problems (Kvist *et al.*, 1990).

Results obtained by the assays such as SCSA, Tunel and Comet can be manipulated by experimental variation of the zinc-dependent chromatin stability (Björk *et al.*, 2009, Pettersson *et al.*, 2009, Tu *et al.*, 2009) demonstrating an important source for false results as well as support for that the methods primarily measure the accessibility of the sperm DNA rather than a primary DNA damage.

Conclusions

If performed with the best available techniques by staff with proper training in laboratories employing internal quality control and participating in external quality assessment programs, basic semen analysis can provide valuable information about the man and his reproductive organs, as well as prognostic information. Still, the results of semen analysis should always be interpreted including patient history as well as the results of a physical examination and other laboratory investigations.

Sperm DNA integrity tests, although correlated with infertility treatment problems, do not yet provide a useful prognostic tool for the individual couples. None of the developed methods have been validated taking into consideration factors causing variation in chromatin stability.

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Apoptoza spermijev in antioksidativna moč semena pri diagnostiki moške neplodnosti

Sperm apoptosis and seminal antioxidant power in the diagnosis of male infertility

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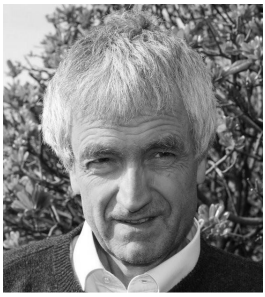

V Parizu se je izpopolnjeval iz andrologije med 1994 in 1996. Od aprila 1995 je zaposlen na Kliničnem oddelku za reprodukcijo na Ginekološki kliniki Unvierzitetnega kliničnega centra Ljubljana, kjer raziskuje in zdravi moško neplodnost ter vodi laboratorij za andrologijo. Od leta 2004 je docent iz predmeta ginekologije in porodništva.

Povzetek

Poleg klasičnega spermiograma imajo funkcionalni testi dvojni namen: spoznavati patofiziologijo poškodbe spermija in razvijati specifična zdravljenja. V raziskavi smo ocenili semenske vzorce 177 moških iz neplodnih parov. V izvornem semenu smo opravili klasično analizo semena in merili skupno antioksidativno zmogljivost (TAC), encima glutation-peroksidazo (GPx) in superoksid-dismutazo (SOD) in selen (Se). V istem semenu, pripravljenem po metodi gradientov gostot, smo s pretočno citometrijo določili označevalce apoptoze, mitohondrijski membranski potencial (Δ_m) in fragmentacijo DNK.

Ugotovili smo statistično značilno povezavo med klasičnimi značilnostmi spermijev in odstotkom spermijev z normalnim Δ_m in s fragmentirano DNK (P<0.001) in s Se (P=0.001). Ugotovili smo negativno korelacijo med fragmentacijo DNK in Δ_m (r=0.725, P<0.001) in pozitivno korelacijo med SOD in GPx (r=0.303, P=0.027) in Se (r=0.513, P<0.001) in med Δ_m in Se (r=0.293, P=0.041).

Fragmentacija DNK je pozitivno povezana z dolžino abstinence (P=0.011) in z volumnom moda (P=0.006). Mitohondrijski potencial je pozitivno povezan z volumnom moda (P=0.017). TAC je negativno povezan s starostjo (P=0.026), GPx negativno (P=0.002) z varikokelo (412.6 ± 102.6 brez varikokele proti 283.4 ± 116.9 ob prisotni varikokeli) in Se pozitivno s trajanjem abstinence (P=0.024).

Poleg označevalcev apoptoze so lahko tudi antioksidanti v semenski tekočini pokazatelji kakovosti spermijev. Skupno določanje klasičnih značilnosti in označevalcev apoptoze ter antioksidantov naj bi omogočalo natančnejšo diagnostiko moške neplodnosti.

Abstract

Beside classical sperm analysis, functional tests have emerged with double goal: to understand the pathophysiology of sperm damage and to develop specific therapies. Semen samples from 177 men from infertile couples were evaluated. The study involved the determination of classical sperm characteristics and total antioxidant capacity (TAC), enzymes glutathione peroxidase (GPx) and superoxide dismutase (SOD), and selenium (Se) in neat seminal plasma and sperm apoptosis markers i.e. mitochondrial membrane potential (Δ_m), DNA fragmentation in density gradient prepared semen using flow cytometry.

A significant association existed between classical sperm characteristics and sperm with normal Δ_m and DNA fragmentation (P<0.001), and with Se (P=0.001). DNA fragmentation correlated with Δ_m (r=0.725, P<0.001) negatively and SOD with GPx (r=0.303, P=0.027) and Se (r=0.513, P<0.001), and with Se (r=0.293, P=0.041) positively.

DNA fragmentation correlated with duration of abstinence (P=0.011) positively and testicular volume (P=0.006) negatively. Mitochondrial potential correlated positively with testicular volume (P=0.017). TAC correlated with male age (P=0.026) negatively, GPx with varicocele (P=0.002) negatively (412.6 ± 102.6 without varicocele vs. 283.4 ± 116.9 with varicocele), and Se correlated with duration of abstinence (P=0.024) positively.

Besides markers of sperm apoptosis, antioxidants in the seminal plasma may be indicators of sperm quality. Combined assessment of classical sperm characteristics, apoptosis markers and antioxidants might help us to improve the diagnosis of male infertility.

Ključne besede: Fragmentacija DNK, GPx, klasične značilnosti semena, mitohondrijski potencial, moška neplodnost, Se, SOD, TAC.

Key words: DNA fragmentation, Gpx, sperm classical characteristics, mitochondrial potential, male infertility, Se, SOD, TAC.

Izhodišče

Ob klasičnih značilnostih lahko kakovost spermijev dodatno ugotavljamo z analizo sprememb v fragmentaciji DNK (Benchaib in sod., 2007) in v potencialu mitohondrijske membrane (Marchetti in sod., 2002).

Povečani kisikovi radikali (ROS) so povezani z nižjim mitohondrijskim potencialom in s povečano fragmentacijo DNK. V semenski tekočini so povišani ROS povezani z nižjo stopnjo oploditve v klasičnem IVF (Agarwal in sod., 2005). V naši raziskavi smo ROS določili posredno z merjenjem skupne antioksidativne zmogljivosti (TAC), encima glutation-peroksidazo (GPx) in superoksid-dismutazo (SOD) in selena (Se). Namen raziskave je bil preiskovati: 1. kakšne so povezave med označevalci apoptoze in antioksidanti in klasičnimi značilnostmi spermijev; 2. kako so označevalci apoptoze in antioksidanti med seboj povezani in kateri dejavniki vplivajo nanje.

Metode dela

Preiskovanci

Od junija 2007 do junija 2009 smo v ambulantah Centra za andrologijo Ginekološke klinike, Univerzitetni klinični center Ljubljana v raziskavo vključili zaporednih 177 moških partnerjev iz neplodnih parov.

Klasična ocena kakovosti semena in merjenje skupne antioksidativne zmogljivosti (TAC), encimov SOD in GPx in selena v izvornem semenu

Seme smo analizirali po priporočilih Svetovne zdravstvene organizacije (WHO) glede na volumen, koncentracijo spermijev (normalna = ≥20x10^6 spermijev/mL), hitro gibljivost v smeri ali gibljivost »a« (normalna = ≥25 %) in normalno morfologijo, označeno s strogimi kriteriji (normalna = ≥14 %). Če so bile vse značilnosti normalne, smo moške diagnosticirali kot normospermike, vse ostale pa kot moške z oligoastenoteratozoospermijo (OAT).

TAC in SOD in GPx aktivnosti smo merili z uporabo kolorometričnih testov - TAC, RANSOD and RANSEL kiti (Randox Laboratories Ltd, Crumlin, UK). Selen smo določili z atomskim absorbcijskim spektrometrom Varian Spectr AA800 (Mulgrave, Australia).

Priprava spermijev in semenske tekočine

Seme smo pripravili na koncentracijskem gradientu PureSperm 100%/40% (Nidacon, International AB, Sweden). Po 30-min centrifugiranju pri 160 *g* smo spodnjo frakcijo vzorca sprali z 2 mL gojišča Sperm Prep (MediCult, Jyllinge, Denmark), ponovno centrifugirali 10 min pri 220 *g* in usedlino resupendirali v 0,5 mL Sperm Prep medija. Vzorec tako pripravljenega semena s končno koncentracijo 1x10^6/mL smo uporabili za določitev označevalcev apoptoze s pretočno citometrijo.

Merjenje označevalcev apoptoze v pripravljenem semenu

Apoptozo spermijev smo določili na dva načina: z merjenjem mitohondrijskega membranskega potenciala (Δ_m), za kar smo uporabili karbocianatno barvilo $DiOC_6(3)$ in z merjenjem fragmentirane DNK s TUNEL tehniko. S pretočnim citometrom FACSCalibur (Becton Dickinson) smo izmerili intenziteto fluorescenčne svetilnosti v vzorcih.

Statistika

S Spearmanovim testom smo iskali korelacije med klasičnimi značilnostmi semena in označevalci apoptoze in antioksidanti. Z Mann-Whitneyevim *U* testom in analizo variance smo dodatno analizirali, kako se označevalci apoptoze in antioksidanti razlikujejo glede na to, ali je seme normalno ali ne. Z analizo linearne regresije smo ugotavljali, kateri dejavniki vplivajo na označevalce apoptoze in antioksidante. Testirali smo naslednje spremenljivke: starost moškega, trajanje abstinence, anamnezo retiniranega moda, volumen mod, prisotnost varikokele.

Rezultati

Povezava med klasičnimi značilnostmi spermijev (koncentracija, hitra gibljivost, morfologija) in označevalci apoptoze (delež spermijev z normalnim $\Delta\psi_m$ in s fragmentirano DNK) ter antioksidanti.

Med klasičnimi značilnosti semena in označevalci apoptoze je obstajala močna (P<0.001) pozitivna korelacija med značilnostmi in deležem spermijev z normalnim potencialom ali negativna med značilnostmi in deležem s fragmentirano DNK. Visoka pozitivna povezava je obstajala tudi med značilnostmi in Se (P=0.001). Encim SOD pa je bil povezan le s koncentracijo spermijev (r=0.311, P=0.023).

Razlike med označevalci apoptoze in antioksidanti glede na to, ali je seme normalno ali ne

Označevalca apoptoze sta bila pomembno različna med moškimi z normozoospermijo in moškimi z OAT (P<0.001). Ti dve skupini moških sta se tudi razlikovali glede na količino Se (P=0.003).

Povezave med označevalci apoptoze in antioksidanti

Fragmentacija DNK je bila povezana z mitohondrijskim potencialom (r=0.725, P<0.001).

SOD je bil povezan z GPx (r=0.303, P=0.027) in Se (r=0.513, P<0.001). Mitohondrijski potencial pa je bil povezan še s Se (r=0.293, P=0.041).

Dejavniki, ki vplivajo na označevalce apoptoze in antioksidante

Fragmentacija DNK je bila pozitivno povezana s trajanjem abstinence in negativno z volumnom moda. Mitohondrijski potencial je bil pozitivno povezan s selenom in z volumnom moda. TAC je bil negativno povezan s starostjo, GPx negativno z varikokelo in Se pozitivno z abstinenco (Tabela 1).

Tabela 1. Dejavniki, ki vplivavo na označevalce apoptoze in antioksidante.

	Starost moškega	Trajanje abstinence	Volumen moda	Varikokela (odsotna n=114/prisotna n=57)
Frag. DNK (%)	Ni povezave	P=0.011	P=0.006	Ni povezave
$\Delta\psi m$ (%)	Ni povezave	Ni povezave	P=0.017	Ni povezave
TAC (mmol/L)	P=0.026	Ni povezave	Ni povezave	Ni povezave
GPx (U/mL x 10^{-3})	Ni povezave	Ni povezave	Ni povezave	412.6/283.4, P=0.002
Se (µg/L)	Ni povezave	P=0.024	Ni povezave	Ni povezave

Diskusija

Potrdili smo povezavo med označevalci apoptoze spermijev s klasičnimi značilnosti semena. Označevalci apoptoze so močno povezani s tremi značilnostmi, kar je skladno z dozdajšnjimi publikacijami. Odstotek spermijev z normalnim Δ_m je pri normozoospermikih približno 70% in se zniža na 50% pri OAT bolnikih (Zorn in sod., 2009). Povprečna fragmentacija DNK v normalnem semenu je 18% in se zviša za polovico pri OAT. Med TAC, GPx in klasičnimi značilnostmi spermijev nismo našli povezav, kar je primerljivo s podatki iz literature, kjer velikokrat primerjajo seme plodnih s semenom neplodnih moških. Le selen je bil povezan s tremi značilnostmi spermijev; kar potrjuje hipotezo, da selen varuje pred oksidativno okvaro DNK. Aktivnost encima superoksid-dismutaza je bila povezana s koncentracijo spermijev. Razlaga te povezave je predvsem v tem, da se SOD ne izloča samo iz prostate, ampak tudi iz samega spermija: če je koncentracija spermijev večja, je večja tudi koncentracija SOD. Druga razlaga pa lahko poudarja pomen SOD kot glavnega antioksidativnega encima: če je koncentracija SOD večja, je večja tudi antioksidativna moč in koncentracija spermijev.

Povezave med mitohondrijskim potencialom in fragmentacijo DNK in med SOD in GPx in selenom so že znane. Ni pa še opisana povezava med mitohondrijskim potencialom in selenom.

Povezava volumna moda z mitohondrijskim potencialom in s fragmentacijo DNK potrjuje, da je apoptoza vpletena v spermatogenezo, in da sta oba označevalca primerna pri ocenjevanju kakovosti spermijev. Dokazuje tudi, da je merjenje volumna mod pomemben kriterij pri postavitvi diagnoze moške neplodnosti. Ena izmed današnjih razlag, zakaj varikokela učinkuje negativno na kakovost semena, je povečan oksidativni stres. Našli smo negativno povezavo med varikokelo in aktivnostjo encima GPx. Mehanizem, zaradi katerega se ob varikokeli dvigne količina ekstracelularnega encima GPx, ki ga izloča nadmodek, je neznan.

Povezava fragmentacije DNK s trajanjem abstinence odpira nove možnosti pri naročanju bolnikov za oddajo semena ali za svetovanje neplodnim parom. WHO priporoča, da je idealno trajanje vzdržnosti 2 do 5 dni; pri bolnikih z visoko fragmentacijo DNK svetujemo, da je vzdržnost čim krajša. Prav tako neplodnim parom priporočamo pogoste spolne

odnose. Količina selena v semenu se z vzdržnostjo sicer dviguje, a to ne učinkuje na fragmentacijo DNK.

Nekateri avtorji so ugotovili negativno povezavo med starostjo moškega in kakovostjo semena; eden mehanizmov poslabšanja je povečana apoptoza predvsem nenormalne DNK. Ugotovili smo, da je s povečano starostjo skupna antioksidativna zmogljivost nižja.

Ob pregledu rezultatov te raziskave menimo, da je mehanizmov, s katerimi apoptoza in oksidativni stres negativno vplivata na kakovost spermijev, več, in da učinkujejo ločeno preko poti, ki jih še ne poznamo. Analiza podatkov lahko vodi do novih patofizioloških hipotez in načrtovanja specifičnih ukrepov oz. zdravljenj.

Vsekakor je pri diagnostiki moške neplodnosti danes pomembno klasični spermiogram dopolniti z določanjem označevalcev apoptoze in antioksidantov. Z golo uporabo spermiograma tvegamo, da ocenimo nenormalne semenčice za normalne semena, kar ima lahko usodne posledice na izid neposrednega vnosa spermija v citoplazmo jajčne celice (ICSI). Poleg spermiograma smo na podlagi ugotavljanja apoptoze spermijev in antioksidantov odkrili, da je približno 30% spermiogramov, ki bi jih ocenili kot normalne, v resnici nenormalnih. Prav tako v skupini nenormalnih spermiogramov lahko ugotovimo, da ima več kot tretjina moških normalno kakovost semena in ne potrebuje metode ICSI. Napredek v tehnologiji nam z razvojem pretočne citometrije omogoča hiter, natančen in poceni dostop do pomembnih preiskav.

Več raziskav tudi kaže na pomembnost določanja označevalcev apoptoze in antioksidantov pri napovedi za nosečnost: pri nenormalnih vrednostih antioksidantov, fragmentacije DNK in mitohondrijskega potenciala je tveganje za slab razvoj zarodkov oz. spontani splav večje (GilVilla in sod., Chabory in sod., 2009). Zato menimo, da si poleg spermiograma testi za apoptozo in oksidativni stres spermijev zaslužijo primerno mesto pri rutinski obravnavi semena in neplodnega moškega.

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Zakaj in kako izbrati najboljši spermij za ICSI?

Why and How to choose the best spermatozoon for ICSI?

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Povzetek

Izhodišča

Z našo raziskavo smo želeli določiti spermije z največjo oploditveno sposobnostjo in razvoja zarodka tik pred vnašanjem v postopku ICSI.

Raziskava

Prospektivna analiza 342 spermijev 32-ih parov z moškim vzrokom neplodnosti, vključenih v postopek ICSI.

Material in metode

S pomočjo visoke povečave (x 6100) (**Slika 1**) smo določili točkovni sistem za izbiro optimalnega spermija pred vnašanjem v postopku ICSI (**Slika 2**).

Rezultati

Statistična analiza je temeljila na osnovi seštevka ocenjevalnih točk spermija: normalna glava = 2 točki, odsotnos vakuol = 3 točke in = normalna baza = 1 točka. Skupni seštevek 6 točk predstavlja morfološko najboljši spermij. Ti kriteriji vplivajo na izzid postopka ICSI.

Glede na točkovni sistem smo spermije razdelili v tri razrede: I = 6-4 točke, II = 3-1 točke in III = 0 točk. Dobili smo statistično pomembno razliko v stopnji oploditve: 92 od 110 (83.6%), 124 od 181 (68,5%) in 29 od 51 (56,9%) (P<0.001, hi kvadrat 14,14).

Pri vnosu spermijev s seštevkom 0 je bila stopnja zanositve nižja in ni prišlo do razvoja ekspandirane blastociste.

Ob tem smo ugotovili statistično pomembno razliko v stopnji ekspandiranih blastocist: 15 od 119 (13.6%), 14 od 181 (7.7), 0 od 51 (0%) (P<0.001, chi square 8.6).

Zaključek

Naša točkovni sistem ocenjevanja spermijev omogoča izbiro najboljšega spermija za postopek ICSI glede na njegovo oploditveno sposobnost in embriogenezo. Tak postopek - SICSI (Scored Intra Cytoplasmic Sperm Injection) izboljša rezultate ICSI postopka (**Slika 3**).

Abstract

Objective

The aim of this study was how to choose the best spermatozoon just before its injection with the highest predictive fertilizing potential and early embryo development during intracytoplasmic sperm injection (ICSI).

Design

Prospective analysis of 342 spermatozoa from 32 couples with male factor infertility referred for ICSI.

Material and methods

Using a high power magnification (x6100) (**Fig. 1**) led us for defining a sperm scoring system for choosing the optimal spermatozoon prior to the injection in ICSI (**Fig. 2**). A detailed classification scoring scale was established for this choice

Results

Our statistical analysis was done according to the following calculated formula for the spermatozoon: Scoring system: normal head = 2 points, lack of vacuole = 3 points, and normal base = 1 point. Total score of 6 points represents morphologically "normal top" spermatozoon, calculated with the major criteria affecting the outcome of ICSI.

Our scoring system of three classes I, II and III (I = 6-4 points, II = 3-1 points and III = 0 points) revealed a statistically significant difference in the fertilization rate: 92 out of 110 (83.6%), 124 out of 181 (68.5%), and 29 out of 51 (56.9%), respectively. (P< 0.001; chi square=14.14)

When spermatozoa with score= 0 were injected a lower fertilization rate was noticed and none expanded blastocyst was developed.

We also obtained a statistically significant difference in the expanded blastocysts rate: 15 out of 110 (13.6%), 14 out of 181 (7.7%), and 0 out of 51 (0%), respectively (P< 0.01; chi square = 8.6).

Conclusion

Our formula scoring spermatozoa provides allows the best spermatozoon to be chosen for ICSI regarding its fertilizing potential and its contribution to embryogenesis. This Scored Intra Cytoplasmic Sperm Injection (SICSI) improves the success rates of the individual ICSI cycle (**Fig. 3**).

Ključne besede: Baza, blastocista, glava, ICSI, IMSI, optimalen spermij, oploditev, SICSI, vakuola.

Key words: Base, blastocyst, head, ICSI, IMSI, optimal spermatozoon, fertilization, SICSI, vacuole.

Fig. 1. Spermatozoa at magnification x6600.

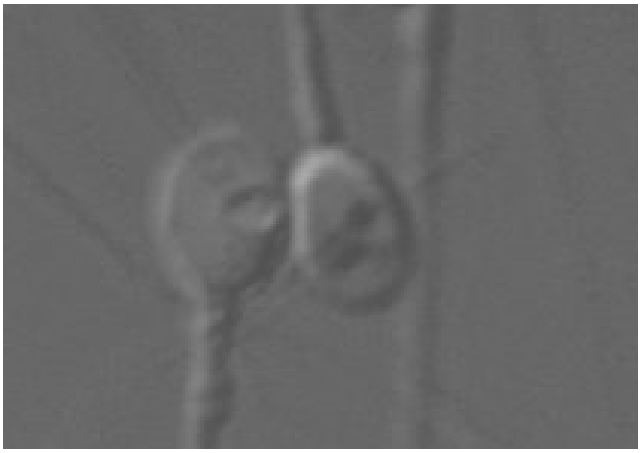


Fig. 2. HBV scoring.

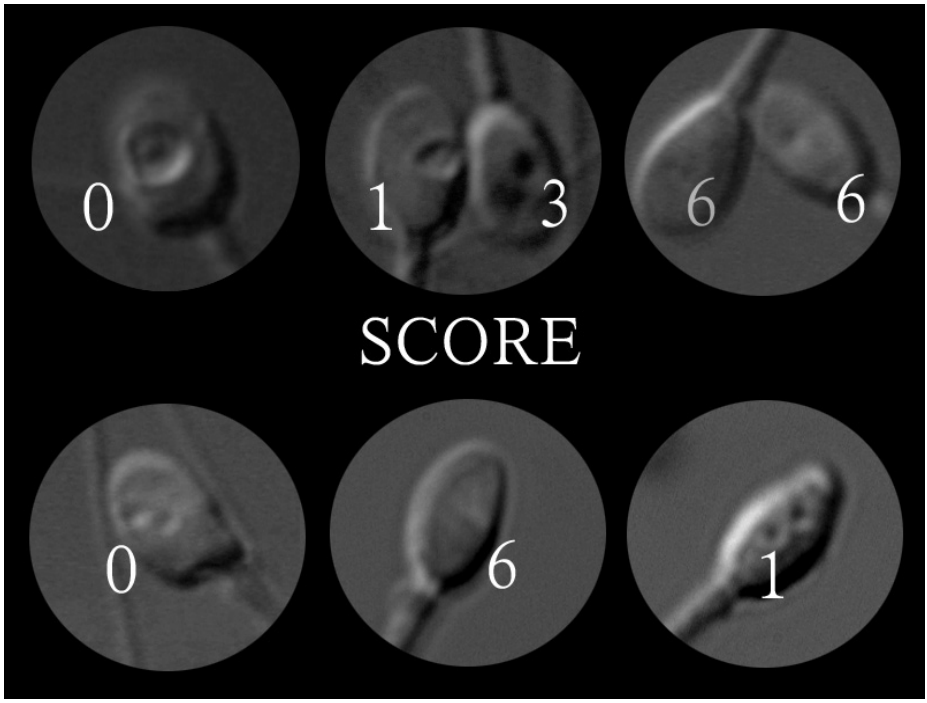
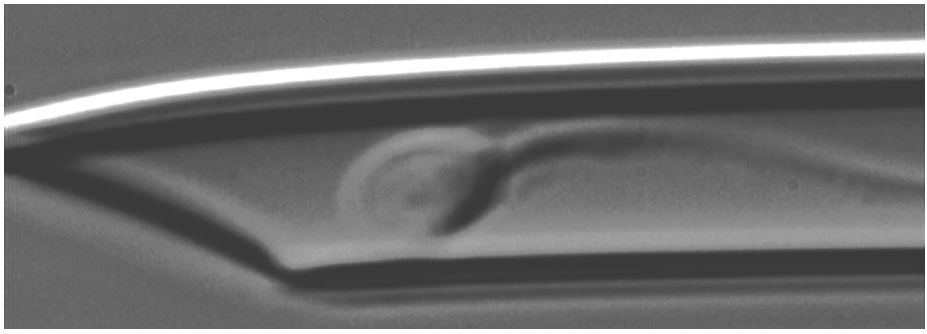


Fig. 3. SICSI.



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Zdravljenje moške neplodnosti z gonadotropini

Gonadotropin treatment for infertility in men

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Povzetek

FSH ima pomembo vlogo v reprodukciji človeka. Že v fetalnih in neonatalnih razvojnih stopnjah vpliva na rast Sertolijevih celic, v puberteti pa na mitotično delitev spermatogonijev in podpira diferenciacijo do stopnje okroglih spermatid. Ugotovljena fiziološka vloga FSH-ja v spermatogenezi je privedla do različnih poskusov zdravljenja nepojasnjene oligospermije. Znano je, da je zdravljenje z gonadotropini zelo učinkovito v primeru hipogonadotropičnega hipogonadizma, kar pogosto privede do popolne normalizacije spermatogeneze. Enak princip zdravljenja so uporabili tudi pri zdravljenju oligospermije, z namenom da bi povečali število spermijev. Dobljeni rezultati so nasprotujoči. Znano je, da zdravljenje s FSH ni učinkovito pri vseh primerih oligospermije, temveč le v določeni podskupini. Potrebno bi bilo postaviti kriterije za izbor tistih moških, ki se odzivajo na zdravljenje. V prispevku bomo navedli pregled literature in naše izkušnje z zdravljenjem oligospermije s FSH.

Abstract

FSH plays a crucial role in human reproduction. Already in the fetal and neonatal developmental stages, FSH activates the proliferation of the Sertoli cells, and during the pubertal phase it induces the mitotic activity of the spermatogonia and supports cellular differentiation until the round spermatid stage. This physiological role in spermatogenesis has induced various attempts to treat idiopathic oligozoospermic men with FSH. It is well known that treatment with gonadotropins is very effective in subjects affected by hypogonad-

otropic hypogonadism, often leading to the restoration of normal spermatogenesis. The success of FSH treatment in these men has brought the utilization of the same therapy in infertile oligozoospermic subjects, aimed at obtaining a quantitative increase in sperm count. However, the results obtained so far are still controversial. What is however well established is that FSH treatment is not useful in all, unselected cases of oligozoospermia, but only in a subgroup of them. Selection criteria are therefore necessary to individuate men who will respond to therapy. In this paper, the literature is reviewed and the authors' experience on using FSH treatment in oligozoospermic subject is discussed.

Ključne besede: Gonadotropini, nepojasnjena oligozoospermija, zanositev, spermatogeneza, število spermijev, citologija mod.

Key words: Gonadotropins, idiopathic oligozoospermia, pregnancy, spermatogenesis, sperm count, testicular cytology.

Introduction

Follicle-stimulating hormone (FSH) is one of the major hormones produced by the anterior pituitary gland. It is known that FSH in the fetal and neonatal developmental stages activates the proliferation of the Sertoli cells and successively, in the pubertal phase it induces the mitotic activity of the spermatogonia and supports cellular differentiation, by meiotic divisions, until the round spermatid stage.

It is well known that treatment with gonadotrophins is very effective in subjects affected by hypogonadotrophic hypogonadism (Matsumoto *et al.*, 1986, Mastrogiacomo *et al.*, 1991, Burgues and Calderon, 1997) often inducing the restoration of normal spermatogenesis and spontaneous pregnancy. This success has led to application of the same therapy in oligozoospermic men, aimed at obtaining a quantitative increase in sperm count. However, the results obtained so far are still controversial. In fact, some studies have demonstrated that FSH treatment is able to increase the spermatogonial population, sperm concentration and pregnancy rate in oligozoospermic subjects with normal plasma concentration of gonadotrophins (Acosta *et al.*, 1992; Bartoov *et al.*, 1994; Sigg and Baciu, 1994; Merino *et al.*, 1996; Glander and Kratzsch, 1997; Strehler *et al.*; 1997), while other authors did not support these data denying the worth of FSH therapy on seminal parameters (Acosta *et al.*, 1991; Matorras *et al.*, 1997; Kamischke *et al.*, 1998; Ben-Rafael *et al.*, 2000). These differences can, in part, be justified by various factors, such as criteria adopted in the selection of patients, the interpretation of seminal parameters, the treatment dose, and its duration. Another important consideration rises from the pathogenesis of the testicular damage that is responsible for the reduced sperm concentration in the ejaculate. Furthermore, the reduced sperm production may be associated with different testicular alterations, such as hypospermatogenesis and maturation disturbances at the spermatogonial, spermatocytic or spermatidic level (Foresta and Varotto, Foresta *et al.*, 1992; Foresta *et al.*, 1995). The most frequent criteria utilized for the selection of patients to be treated with FSH is represented only by the normality of plasma gonadotrophin concentrations, while the usefulness of the treatment is evaluated exclusively on the basis of sperm concentration or pregnancy rate. Other studies have studied different predictive parameters able to identify the subjects with

potential responsiveness to FSH treatment, such as evaluation of testicular cytology or inhibin B plasma concentrations. With this aim we performed some studies over the last few years that will be discussed below.

FSH treatment and testicular cytology

Tubular function was studied using testicular fine needle aspiration cytology (FNAC) (Foresta and Varotto, Foresta *et al.*, 1992; Foresta *et al.*, 1995; Foresta *et al.*, 1998) in order to evaluate the specific alteration responsible for reduced sperm production. In this study, 60 oligozoospermic subjects with normal FSH plasma concentrations (< 7 IU/L) were treated with highly purified FSH (75 IU on alternate days for 3 months). This treatment allowed the identification of a subgroup of men who had at least doubled their sperm concentration (responders). Interestingly, in non responder subjects FNAC performed before the treatment showed hypospermatogenesis associated with spermatidic maturation defects, whereas in the group of responders we observed hypospermatogenesis with a normal maturation process. The cytological analysis was repeated after FSH treatment and showed an increased number of spermatogonia, spermatocytes and spermatids in all the subjects. Nevertheless, only in the responder group there was an activation of the spermiogenic process, with a consequent increase in both the number of intra-testicular spermatozoa and sperm concentration in semen.

The results of this first study highlighted a prevalent effect of FSH on pre-meiotic and meiotic cells and led us to conclude that the choice of treatment with FSH should depend on the knowledge of the specific tubular alteration that induced oligozoospermia. Only in the cases in which oligozoospermia was sustained by hypospermatogenesis without maturation alterations, FSH treatment induced a significant increase in the concentration of ejaculated spermatozoa. Instead, in the presence of hypospermatogenesis associated with post-meiotic alterations, FSH further amplified the maturation difficulties in the final phases of spermatogenesis without a significant increase in the sperm count.

FSH and inhibin B plasma concentrations

Another parameter that was considered to identify subjects with potential responsiveness to FSH treatment was inhibin B plasma concentrations, a well known marker for tubular function secreted by Sertoli cells (Foresta *et al.*, 2000). Oligozoospermic men were divided into 3 groups based on basal FSH and inhibin B plasma levels: group A (normal FSH and inhibin B) characterized by moderate hypospermatogenesis sometimes associated to a partial spermatidic arrest; group B (high FSH and normal inhibin B) characterized by hypospermatogenesis associated or not to spermatogonial-spermatocytic arrest; group C (elevated levels of FSH and low levels of inhibin B) characterized by severe hypospermatogenesis. At the end of the treatment, a significant increase in ejaculated sperm concentration was observed only in oligozoospermic subjects with normal basal FSH and inhibin B plasma levels (the subjects from group A), showing a testicular cytological picture of moderate hypospermatogenesis. In the remaining patients from group A (hypospermatogenesis associated with maturation defects) and in all subjects from groups B and C, there was no

significant increase in the sperm count. These data further suggest that FSH treatment may have a role in oligozoospermic subjects only when spermatogenetic alterations consist in germ cell depopulation without maturation disturbances and with normal FSH concentrations. Thus again, the knowledge of the tubular status seems to represent a condition able to predict the potential success of the treatment with FSH (**Table 1**). Also, the elevated basal levels of FSH, independently of inhibin B concentrations seem to be a negative predictive factor for the usefulness of the treatment.

Table 1. *Clinical characteristics in responders and non responders to FSH treatment (Foresta et al., 2000).*

	Semen analysis Sperm count	**FSH** plasma levels	**Inhibin B** plasma levels	**Spermatogenic alteration** FNAC/Biopsy
Responders	Oligozoospermia	Normal	Normal	Hypospermatogenesis without maturation arrest
Non Responders	Oligozoospermia	Normal	Normal	Hypospermatogenesis with maturation arrest
Non Responders	Oligozoospermia	High	Normal	Hypospermatogenesis with or without maturation arrest
Non Responders	Oligozoospermia	High	Low	Hypospermatogenesis with or without maturation arrest

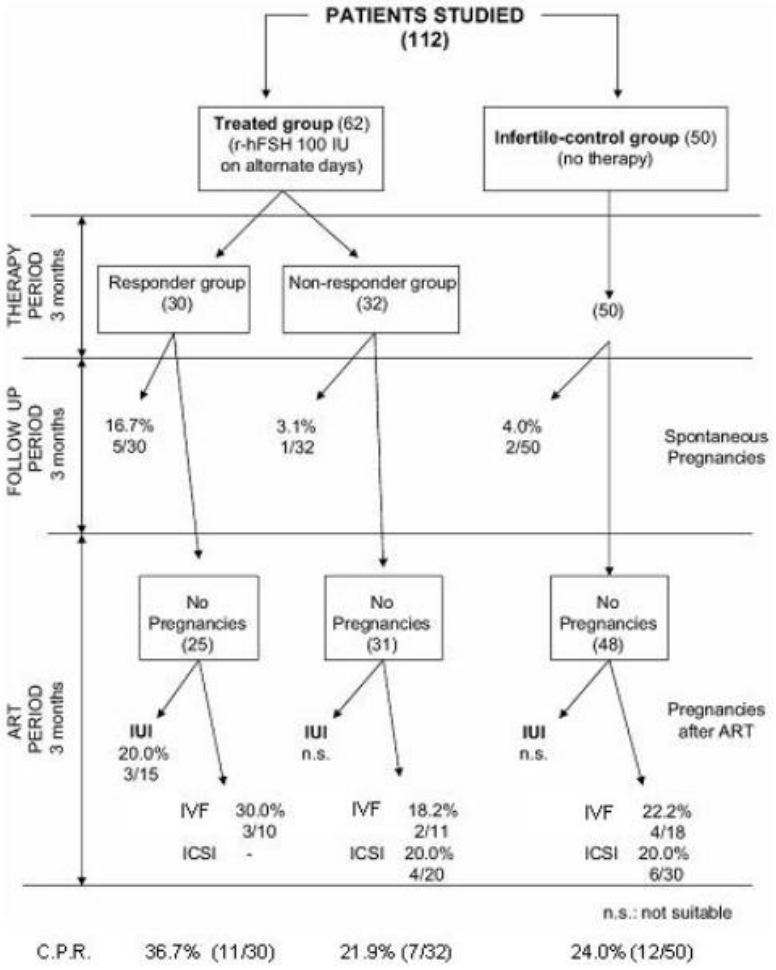
Highly purified vs. recombinant FSH

All these data obtained with highly purified FSH were confirmed with recombinant FSH (r-hFSH) (Foresta *et al.*, 2002). We evaluated a well selected group of 45 subjects with tubular, seminologic and hormonal characteristics that were compatible with those of subjects previously identified as responsive to highly purified FSH. Thirty oligozoospermic patients, who had hypospermatogenesis without maturation defects and had normal levels of FSH, were treated with r-hFSH, 15 patients were not treated. Half of the patients received a dose of 50 IU r-hFSH while the other 15 were treated with 100 IU. Both groups were given the doses every other day for 3 months. At the dose of 50 IU, the treatment with recombined FSH induced a significant increase in the sperm concentration in only 2 of the 15 patients, similarly to the control group. On the contrary, among the subjects treated with 100 IU, 11 of the 15 showed at least a doubling of sperm concentrations with respect to basal values. The results of this study showed that the treatment with a dose of r-hFSH 100 IU every other day for 3 months is able to increase the spermatogonial population and the sperm concentration in patients with idiopathic oligozoospermia, normal FSH plasma levels and testicular cytology characterized by hypospermatogenesis without maturation arrest.

Effects of FSH treatment on pregnancy rates

To evaluate the usefulness of the treatment with FSH in terms of the pregnancy rate, we followed a group of subjects treated with recombinant FSH for a period of 6 months after suspension of a 3-month FSH therapy. In this study (Foresta *et al.*, 2005) we evaluated 112 oligozoospermic patients randomized into two groups: 62 subjects treated with r-hFSH 100 IU every other day for 3 months and 50 subjects who were not treated and used as a control group (**Figure 1**).

Figure 1. *Description and results of the study of oligozoospermic patients treated (responders and non-responders) with recombinant human FSH (r-hFSH) and control subjects followed up for 6 months after treatment (adapted from 20). ART = assisted reproductive technology; CPR = cumulative pregnancy rate; IVF = in-vitro fertilization; ICSI = intracytoplasmic sperm injection; IUI = intrauterine insemination; n.s. = not suitable.*



Semen analysis was performed in all subjects at the end of this treatment period and after the following 3 months. During the three months after the suspension of the therapy, among treated subjects we registered 5 spontaneous pregnancies in responder subjects and one pregnancy in the non responders (respectively 16.7% and 3.1%), while among untreated subjects we observed two pregnancies (4.0%). In the responder group, 15 patients were able to undergo intra-uterine insemination that permitted 3 further pregnancies (20%) while another 10 patients underwent in vitro fertilization (IVF), with 3 pregnancies (30%). In subjects from the other two groups it was not possible to proceed in any cases with intra-uterine insemination due to very poor seminal parameters. Six months after the suspension of the treatment, the cumulative results in terms of pregnancy rate were: 36.7% in responders, 21.9% in non-responders and 24.0 % in the untreated group. In addition to the higher pregnancy rate obtained in responder subjects after FSH treatment, the therapy also induced an improvement of the seminal parameters. Importantly, this improvement allowed the same subjects to undergo assisted reproduction techniques that were less invasive for female partners (intra-uterine insemination), or to use the natural capacity of spermatozoa to fertilize by the use of IVF instead of ICSI. Also on these results are based the conclusions of the last Cochrane Review on the use of gonadotropins in the treatment of male infertility (Attia *et al.*, 2006). This meta-analysis highlights that in infertile subjects, FSH treatment induces a significant increase in terms of percentages of pregnancy rate with respect to control subjects.

Conclusions

FSH treatment may represent a valid tool for infertile men. However, it should be performed in selected patients utilizing some predictive parameters able to identify a priori responder subjects with high probability. The choice of treatment with FSH should never be taken away from the knowledge of the specific tubular alteration that induced oligozoospermia. In particular, the therapy with FSH is able to stimulate the tubular function by bringing about an increase in the population of spermatogonia. Nonetheless, only in the cases in which oligozoospermia is sustained by hypospermatogenesis without maturation alterations in the germ line, a contemporary significant increase in the concentration of ejaculated spermatozoa is expected. Elevated plasma levels of FSH, independently on the concentration of inhibin B, seem to be a negative predictive factor for the usefulness of FSH treatment. Our data together with the last Cochrane Review on the use of gonadotrophins for idiopathic male factor infertility demonstrate that the treatment with FSH in selected cases is associated with a higher percent of spontaneous and assisted pregnancy in subjects who are responsive to therapy. In addition, the improvement of seminal parameters allows the patients to access to assisted reproduction techniques that are less invasive than those possible before treatment.

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Zdravljenje pozno nastalega hipogonadizma s testosteronom

Androgen replacement treatment of the ageing male

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Povzetek

Pozno nastali hipogonadizem (PNH) je skupek simptomov, ki se pojavljajo pri starejših moških kot posledica usihanja moškega spolnega hormona testosterona. Testosteron je učinkovito zdravilo za nadomestno zdravljenje, ki je indicirano, ko ob kliničnih znakih in simptomih dokažemo še znižane koncentracije testosterona v serumu. Uporabljamo testosteron v gelu, ki se maže na nadlaht vsako jutro, in testosteron undekanoat v obliki depot injekcije, ki se daje globoko intramuskularno na 12 tednov. Oba testosteronska pripravka imata dobre farmakokinetične lastnosti, saj vzdržujeta normalne koncentracije testosterona brez nihanj v supra- in subfiziološka območja. Zdravljenje s testosteronom ugodno vpliva na telesno sestavo (poveča mišično maso in zmanjša količino maščobnega tkiva, predvsem centralno, poveča mineralno kostno gostoto), normalizira spolno funkcijo, razpoloženje in izboljša kognitivne sposobnosti ter nekatere presnovne kazalce ter poveča hematopoezo. Pred uvedbo testosterona je potrebno izključiti rak prostate in poliglobulijo, med zdravljenjem pa redno kontrolirati hematokrit, PSA in prostato. Z našo študijo, ki jo kratko predstavljamo, smo dokazali ugodne učinke NTZ na telesno sestavo in endotelijsko funkcijo pri sladkornih bolnikih tipa 2, pri katerih se PNH pojavlja pogosteje in prej kot pri ostali moški populaciji.

Abstract

Late-onset hypogonadism (LOH) is a cluster of signs and symptoms in males that develops gradually with ageing. The main cause is a gradual decrease in testosterone secretion which starts in late thirties and induces decline in sexual and cognitive functions, lean body mass,

bone mineral density, erythropoiesis and an increase in fat mass and insulin resistance. Approximately 6% to 12% of men older than 40 years have clinical androgen deficiency as defined by recent clinical guidelines (ie, low testosterone levels plus signs and symptoms). Although there are concerns about the risks of testosterone replacement, e.g. increased risk of prostate cancer, its use has increased in the past decade. Low testosterone level and androgen deficiency have been associated with abdominal obesity, diabetes mellitus and prediabetic states (insulin resistance, impaired glucose tolerance, and metabolic syndrome), and dyslipidemia, among other outcomes. In addition, low testosterone level has been associated with increased mortality risk in male veterans. Therefore, the testosterone replacement therapy is justified in LOH under close surveillance of prostate and hematocrit. Two forms of testosterone preparations are available in Slovenia: testosterone gel and testosterone undecanoate depot injections. Both have acceptable pharmacokinetic characteristics.

We conducted a prospective trial in a cohort of type 2 diabetic male patients (33) with LOH who were treated with testosterone undecanoate for 7 months (TRT). The estimated prevalence of hypogonadism in patients with type 2 diabetes attending the Diabetes Outpatient Clinic of UMC Ljubljana was 68.9%. Serum testosterone levels increased as expected. Endothelial function as assessed by the US measurement of the flow-mediated endothelium dependent dilation of the brachial artery (FMD) increased from 4.2±4.5% to 7.4±4.8%, (P=0.009), an increase in lean body mass from 73.9±9.5 to 74.9±9.5kg (P=0.045) and a decrease in total body fat mass from 23.2±5.2 to 22.2±5.6kg (P=0.006) was observed. There were no significant changes in lipid and HbA1c levels, the GLUT4 gene expression in subcutaneous fat tissue decreased. The ageing male symptoms (AMS) score improved significantly. Our trial was the first to examine the influence of TRT on the endothelial function in hypogonadal men with type 2 diabetes. We have shown that TRT significantly improves endothelial function, body composition and symptoms of LOH. In our cohort of patients with type 2 diabetes the parameters of metabolic control did not improve.

Ključne besede: Diabetes, endotelij, nadomestno zdravljenje, pozno nastali hipogonadizem, spolnost, telesna sestava, testosteron.

Key words: Diabetes, body composition, endothelium, late-onset hypogonadism, replacement therapy, sexuality, testosterone.

Uvod

Testosteron je glavni moški spolni hormon, ki ga izločajo Leydigove celice v modih. Testosteron omogoča normalno spolnost (libido, erektilno funkcijo, doživljanje orgazma), je anabolni hormon in povečuje mišično maso in moč, zavira kostno resorpcijo, spodbuja tvorbo kostnine in povečuje mineralno kostno gostoto. Spodbuja eritropoezo v kostnem mozgu, sintezo eritropoetina v ledvicah in povečuje število eritrocitov. Ugodno vpliva na kognitivne funkcije, na razpoloženje in storilnost (Jockenhovel, 2004).

Ugotavljajo, da je pri moških z večjimi vrednostmi testosterona splošna umrljivost in umrljivost za srčno-žilnimi boleznimi za 40 % manjša kot pri tistih z manjšimi (Laughlin *et al*., 2008; Khaw *et al*., 2007).

Če moda ne delujejo zadostno, se ne izloča dovolj testosterona in/ali se ne tvori dovolj semenčic. Tako stanje imenujemo hipogonadizem (Jockenhovel, 2004). Hipogonadizem pri moškem opredelimo kot primarni, sekundarni ali pozno nastali. Vzrok primarnega hipogonadizma je prizadetost mod. Najpogostejše oblike so prirojena ali pridobljena okvara v delovanju mod, Klinefelterjev sindrom (en od 500 rojenih dečkov) in disgenezija gonad.

Vzroki sekundarnega hipogonadizma so okvare v področju hipotalamusa ali hipofize, ki povzročijo motnje v izločanju gonadotropinov. Najpogosteje gre za insuficienco hipofize zaradi hipofiznih tumorjev ali posledice njihovega zdravljenja, idiopatski hipogonadotropni hipogonadizem, ali za redke hipotalamične sindrome.

Pozno nastali hipogonadizem (PNH), ki nastane s staranjem in ga nekateri imenujejo sindrom pomanjkanja testosterona, andropavza (napačno!) ali hipogonadizem v starosti, se začne pojavljati po 50. letu. Njegova pojavnost narašča z vsakim naslednjim desetletjem (Jockenhovel, 2004; Araujo *et al*., 2004; Travison *et al*., 2007).

Pozno nastali hipogonadizem (PNH)

PNH je skupek simptomov, ki se pojavljajo pri starejših moških kot posledica usihanja moškega spolnega hormona testosterona. Večina simptomov je nespecifičnih, saj se lahko pojavljajo tudi pri različnih drugih stanjih ali boleznih. Razdelimo jih v telesne, duševne (čustvene - razpoloženjske in kognitivne - umske) ter spolne (Barrett-Connor et al., 1999; Carnahan and Perry, 2004; Zitzmann et al., 2006). PNH pri moških in menopavza pri ženskah se razlikujeta predvsem po nastanku. Menopavza nastopi hitro, ker jajčniki naenkrat prenehajo izločati estrogene. Zato so simptomi pri ženskah bolj intenzivni, moteči in opazni. Pri moških pa se PNH prikrade zelo počasi in postopoma. Vzrok PNH je zmanjšano izločanje testosterona, ki začne usihati okrog 40. leta (Jockenhovel, 2004; Araujo et al., 2004). Izločanje testosterona se vsako leto zmanjša za 1.6%, prostega testosterona pa za 2 - 3%. Tako so ugotavljali pomanjkanje androgenov in PNH pri 6% moških starih 39 - 70 let (začetek Massachusetts Male Aging Study, n= 1709), po 7 – 10 letih je bila prevalenca v isti populaciji moških (tokrat n=1087) 12.3% (Araujo et al., 2004). Biološki učinki testosterona se zmanjšujejo bolj kot se zmanjšuje koncentracija celokupnega testosterona, ki jo običajno merimo. Tvorbo in izločanje testosterona lahko zavrejo tudi nekatere bolezni (Travison et al., 2007). Preden postavimo diagnozo PNH, moramo vse te bolezni najprej izključiti.

Simptomi in znaki PNH

Telesni simptomi PNH se kažejo z manjšanjem mišične mase in nižanjem mineralne kostne gostote, kar povečuje nevarnost zlomov, zmanjša se tudi mišična moč in koncentracija hemoglobina v krvi, v telesu pa se nabira več maščobe. Moški imajo podobno kot ženske tudi navale vročine s potenjem. Na čustveni ravni se lahko pojavijo depresija, pomanjkanje

volje, nespečnost, občutki tesnobe, razdražljivost, nihanje razpoloženja (Lunenfeld et al., 2005; Zitzmann et al., 2006). Na kognitivni ravni se slabšata spomin in občutek za orientacijo v prostoru, ki je sicer pri moških boljši kot pri ženskah (Barrett-Connor et al., 1999; Cherrier et al., 2003; Carnahan and Perry, 2004). V spolnosti pa se zmanjša libido, manjše je število semenčic v izlivu, zaradi česar se zmanjšuje plodnost (Jockenhovel, 2004; Lunenfeld et al., 2005; Zitzmann et al., 2006). Pojavijo se tudi motnje erekcije, ki pa imajo pri zrelejših moških lahko tudi druge vzroke (ateroskleroza, nevropatija, določena zdravila…). Opazne spremembe se pri nekaterih pojavijo že po 50. letu. Kronični bolniki izločajo toliko manj testosterona, da se izenačijo z 10 let starejšimi zdravimi moškimi, zato se pri njih znaki PNH prej in bolj izrazijo (Travison et al., 2007). Posebno ogrožena skupina so bolniki z metaboličnim sindromom in s sladkorno boleznijo tipa 2. Nekatere prospektivne študije dokazujejo, da je manjše izločanje testosterona pravzaprav vzrok oziroma dejavnik tveganja za razvoj metaboličnega sindroma in sladkorne bolezni tipa 2 (Laaksonen et al., 2004; Kapoor et al., 2005), posebno pri moških z normalnim indeksom telesne mase (Kupelian et al., 2006).

Metabolični sindrom opredelimo z naslednjimi kriteriji po IDF (Mednarodna zveza za diabetes): Izpolnjen mora biti osnovni pogoj, in sicer povečan obseg pasu, ki je merilo visceralne debelosti (Alberti *et al.*, 2006).

Visceralna debelost (obseg pasu ≥ 94 cm za moške in ≥ 80 cm za ženske), in vsaj 2 od naslednjih 4 dejavnikov:

- povečani trigliceridi ≥ 1,7 mmol/L, ali specifično zdravljena hipertrigliceridemija;
- znižani HDL-holesterol: < 1,03 mmol/L pri moških (in < 1.29 mmol/L pri ženskah), ali specifično zdravljena dislipidemija te oblike;
- povečani krvni tlak: sistolični ≥ 130 ali diastolični ≥85 mm Hg, ali že zdravljena hipertenzija;
- povečana koncentracija glukoze v krvi: ≥ 5,6 mmol/L, ali že znana sladkorna bolezen tipa 2.

Pri bolnikih z metaboličnim sindromom pospešeno potekajo procesi aterogeneze, zato sta močno zvečani obolevnost za srčno-žilnimi boleznimi in umrljivost (Eckel *et al.*, 2005). Pri njih se PHG pojavlja že zgodaj in je prisoten pri 30-50% bolnikov ne glede na starost.

- Klinični znaki hipogonadizma pri odraslem moškem
- Zmanjšan libido in slabše ali odsotne erekcije
- Nihajoče razpoloženje, zmanjšane kognitivne sposobnosti (motnje pomnjenja in pozornosti), utrujenost, depresivno razpoloženje in razdražljivost
- Manjša telesna poraščenost, tanjša suha koža s pomanjkljivim izločanjem loja
- Zmanjšana mišična masa in moč
- Zmanjšana mineralna kostna gostota (osteopenija in osteoporoza)
- Povečano kopičenje visceralnega maščevja
- Anemija

Opisanim simptomom se lahko pridružijo še navali vročine, podobni menopavzalnim pri ženskah, ki nastopijo, ko so koncentracije testosterona izrazito nizke (Jockenhovel, 2004; Lunenfeld *et al.*, 2005; Zitzmann *et al.*, 2006).

Pomanjkanje testosterona se pri moških klinično manifestira pri različnih serumskih koncentracijah testosterona z različnimi znaki in simptomi. Najprej se zmanjšata libido in življenjska energija na splošno, pri nižjih koncentracijah testosterona se pogosteje razvije debelost, moški zaznajo depresivno razpoloženje, kognitivni upad, motnje spanja in koncentracije, nastane lahko sladkorna bolezen tipa 2. Šele pri zelo znižanih koncentracijah testosterona se razvijejo erektilna disfunkcija in navali vročine (Zitzmann *et al.*, 2006).

Opredelitev PNH

Pomanjkanje androgenov se klinično manifestira pri različnih ljudeh pri različnih serumskih koncentracijah testosterona. Nadomestno zdravljenje s testosteronom je upravičeno, ko je serumska koncentracija testosterona znižana pod 8 - 10 nmol/L ob prisotnih kliničnih znakih in simptomih hipogonadizma (Rhoden and Morgentaler, 2004; Bhasin *et al.*, Practice Committee of the American Society for Reproductive Medicine, 2006). Testosteron določamo med 7. in 11. uro zjutraj in znižane vrednosti potrdimo vsaj dvakrat. Če je znižan celokupni testosteron, določimo še prosti testosteron v serumu ali slini, ali SHBG (spolne hormone vežoči globulin) in prosti testosteron izračunamo. Vzrok znižane koncentracije testosterona opredelimo z določitvijo gonadotropinov LH in FSH. Izključimo hiperprolaktinemijo (Bhasin *et al.*, 2006). Povečano izločanje PRL namreč zavira hipofizno gonadno os na več nivojih in povzroča hipogonadizem. Z določitvijo gonadotropinov opredelimo, ali je primarno moteno delovanje testisov ali višjih centrov. Za PNH je značilno, da je spremenjeno delovanje hipotalamusa, ki izloča manj gonadoliberina, hipofiza pa posledično manj gonadotropinov LH in FSH, prizadeto pa je tudi delovanje mod zaradi degenerativnih – starostnih sprememb. Gre torej za kombinirano motnjo na primarnem in sekundarnem nivoju (Jockenhovel, 2004). Izločanje in delovanje testosterona motijo tudi zdravila (glukokortikoidi, ki se uporabljajo za zdravljenje nekaterih vnetij, astme, diuretik spironolakton, nekateri anabolni steroidi, blokatorji beta adrenergičnih receptorjev, pomirjevala in druga), zato bolnika povprašamo o uporabi teh zdravil (Lunenfeld *et al.*, 2005).

Nadomestno zdravljenje s testosteronom

Ob pojavu naštetih simptomov je potrebno napraviti diagnostične preiskave. Če ugotovimo hipogonadizem – znižano koncentracijo testosterona v dveh zaporednih določitvah in izključimo druge bolezenske vzroke, bolnika lahko uspešno zdravimo z nadomeščanjem testosterona (Jockenhovel, 2003; MacIndoe, 2003; Mulhall et al., 2004; Lunenfeld et al., 2005). Njegovo raven v krvi spravimo v območje normalnih vrednosti. Pri nas so na voljo injekcije testosteron undekanoata in gel za lokalno aplikacijo z 1% testosteronom. Bolniki prejemajo undekanoat na 3 mesece globoko i.m., gel pa si mažejo na nadlahti vsako jutro. Testosteron undekanoat in testosteron v gelu imata zelo dobre farmakokinetične lastnosti in vzdržujeta koncentracijo testosterona ves čas v normalnih mejah. Odmerjanje gela in razmike med aplikacijo undekanoata individualno prilagodimo vsakemu bolniku. Zdravljenje

izboljša telesno sestavo, kognitivne sposobnosti in spolno funkcijo (Cherrier et al., MacIndoe, 2003; Rhoden and Morgentaler, 2004; Bhasin et al., 2006). Običajno zmanjša tudi depresijo, ki je pogost vzrok samomorov starostnikov. Študije so pokazale, da samomorilnost pri moških s starostjo narašča, posebno po 65. letu. Pri 85-letnih moških so samomori že dvakrat pogostejši kot pri 65-letnih. Pri ženskah enakih starosti je samomorilnost štiri do desetkrat manjša.

Nadomeščanje testosterona povečuje nevarnost razraščanja raka na prostati, ki pred uvedbo nadomestnega zdravljenja ni bil prepoznan. Zavedati se je potrebno, da je rak prostate v starosti najpogostejši rak pri moških. Zato mora bolnik pred tovrstnim zdravljenjem opraviti pregled pri urologu, ki se ga sicer priporoča na nekaj let, ultrazvok prostate, ter določitev specifičnega prostatičnega antigena (PSA). Testosteron spodbuja eritropoezo in povečuje hematokrit. Če hematokrit preseže mejo 0.52, se lahko zamašijo majhne žile ali se razvoje tromboza večjih ven. Prvo leto zdravljenja z nadomeščanjem testosterona določamo PSA in hematokrit vsake tri mesece, nato pa po presoji, obvezno pa vsaj enkrat letno. Enkrat letno mora starejši bolnik imeti tudi digitorektalni pregled, idealno tudi UZ prostate. Letni porast PSA ne sme biti večji od 0.4.

Številne randomizirane, tudi multicentrične študije so dokazale ugodne učinke testosterona na telesno sestavo, mišično moč, mineralno kostno gostoto, erektilno funkcijo, izboljšanje kognitivnih sposobnosti in razpoloženja ter življenjske kakovosti (Lunenfeld *et al.*, 2005; Bhasin *et al.*, 2006; MacIndoe, 2003). Dokazali so tudi, da nadomestno zdravljenje s testosteronom poveča periferno občutljivost za insulin in pri diabetikih zmanjša koncentracijo glikoziliranega hemoglobina HbA1c (Kapoor *et al.*, 2006). Ne pospešuje aterogeneze, nekatere študije poročajo celo o izboljšanju endotelne funkcije.

Glede nadomeščanja testosterona pri moških s PNH se odločamo za vsakega bolnika posebej s tehtanjem dobrobiti in nevarnosti. Predvsem pa pred začetkom zdravljenja najprej skrbno izključimo vse morebitne bolezni, ki bi pri bolniku lahko povzročile zmanjšano tvorbo in izločanje testosterona. Diagnozo PNH namreč postavimo tudi z izključevanjem drugih diagnoz.

Stranski učinki zdravljenja s testosteronom: akne (redko, na začetku zdravljenja), ginekomastija (redko, prehodna), sindrom prekinitev dihanja med spanjem, poliglobulija (Ht > 0.52), kožne reakcije na mestu aplikacije. Ni dokazov, da bi testosteron povzročil nastanek raka prostate, prav gotovo pa spodbuja njegovo rast. Zato je potrebno pred uvedbo testosterona z gotovostjo izključiti subklinične oblike raka prostate z rektalnim pregledom, določitvijo PSA in po potrebi s transrektalno UZ vodeno biopsijo prostate.

Absolutne kontraindikacije za zdravljenje s testosteronom: karcinom prostate, karcinom dojke, poliglobulija. Relativne kontraindikacije so srčno popuščanje, sindrom prekinitev dihanja med spanjem in benigna hipertrofija prostate. Če se poveča zadrževanje tekočine v telesu ali če nastopijo mikcijske motne, testosteron ukinemo.

Vodenje bolnika med zdravljenjem s testosteronom

Pred uvedbo testosterona je potrebno določiti hematokrit, PSA in mineralno kostno gostoto z DXA. Bolnik, ki je starejši od 50 let, mora opraviti pregled pri urologu in po možnosti transuretralni UZ prostate z biopsijo, če je izvid sumljiv. V prvem letu zdravljenja s testosteronom kontroliramo pri bolnikih starejših od 50 let hematokrit in PSA na tri mesece, če vrednosti ne porastejo (Ht čez 0.52, PSA za več kot 0.4/leto), bolnika nato naročamo na kontrole enkrat letno. Vedno mu določimo hematokrit in PSA, opraviti mora tudi pregled pri urologu. Glede na klinične podatke in serumsko koncentracijo testosterona prilagodimo odmerek testosterona v gelu ali razmik med dvema injekcijama testosteron undekanoata - v primeru nižjih koncentracij testosterona interval med dvema injekcijama skrajšamo na 10 tednov, če so koncentracije prevelike, ga podaljšamo na 14 tednov.

Prospektivna študija: Učinki nadomestnega zdravljenja pozno nastalega hipogonadizma s testosteronom pri naših bolnikih z metaboličnim sindromom in sladkorno boleznijo tip 2

Prospective study: Effects of LOH testosterone replacement therapy in patients with metabolic syndrome and type 2 diabetes

Presečne študije so pokazale, da ima do 50% moških s sladkorno boleznijo tipa 2 hipogonadizem. Pomanjkanje testosterona povzroča slabšo urejenost sladkorne bolezni, prezgodnji nastanek srčno-žilnih bolezni, erektilno disfunkcijo in zmanjšanje libida.

Med populacijo moških sladkornih bolnikov tipa 2, ki se kontrolirajo v diabetoloških ambulantah UKC Ljubljana, smo določili prevalenco hipogonadizma. Bolnikom, pri katerih smo potrdili diagnozo hipogonadizem, smo uvedli nadomestno zdravljenje s testosteronom (NTZ), da bi preverili učinke zdravljenja na simptome hipogonadizma, metabolično urejenost (HbA1c, ekspresija gena GLUT4, lipidogram), telesno sestavo in endotelijsko funkcijo.

V raziskavo smo vključili 106 bolnikov moškega spola, starejših od 35 let (povprečno starih 61,2 ± 9,3 let s sladkorno boleznijo tipa 2. V prvem – presečnem delu študije smo preiskovancem določili celokupni testosteron in tiste z vrednostmi < 8 nmol/L opredelili kot hipogonadne. V drugem - prospektivnem delu študije smo 33 naključno izbranim preiskovancem s potrjeno diagnozo hipogonadizma uvedli NTZ in jim pred in 7 mesecev po uvedbi določili kazalce metabolične urejenosti, telesno sestavo s pomočjo dvoenergijske rentgenske absorpciometrije (DXA) in endotelijsko funkcijo z ultrazvočnim merjenem od endotelija odvisne razširitve brahialne arterije (FMD). Pred in med NTZ smo jim odvzeli vzorec podkožne trebušne maščobe za določitev ekspresije gena za glukozni prenašalec GLUT4. Bolniki so obakrat izpolnili tudi AMS vprašalnik o simptomih hipogonadizma. Razlike med rezultati pred in po NTZ smo preučevali z dvosmernim Studentovim t-testom za parne vzorce. Statistično analizo smo opravili s paketom »Statistica«, verzija 7.1 (StatSoft inc. 2005, Tulsa, OK, USA).

Prevalenca hipogonadizma med sladkornimi bolniki tipa 2, ki se kontrolirajo v diabetoloških ambulantah UKC Ljubljana, je 68,9%. Po 7 mesecih NTZ se je povprečna vrednost serumskega testosterona pričakovano povečala. Ob tem je FMD porasla s 4,2±4,5% na 7,4±4,8%, (P=0.009), skupna mršava masa s 73,9±9,6 na 74,9±9,2kg (P=0.045), skupna masa telesne maščobe pa se je zmanjšal s 23,2±5,2 na 22,3±5,6kg (P=0.006). V lipidogramu in koncentraciji HbA1c nismo opažali statistično značilnih sprememb, ekspresija gena za GLUT4 v podkožni maščobi se je zmanjšala. Rezultati AMS vprašalnika so se značilno izboljšali.

Prevalenca hipogonadizma med sladkornimi bolniki tipa 2, starejšimi od 35 let, ki se kontrolirajo v diabetoloških ambulantah UKC, je večja kot v primerljivih tujih študijah. Naša raziskava je prva, ki je proučevala vpliv NTZ na endotelijsko funkcijo pri hipogonadnih moških s sladkorno boleznijo tipa 2. Potrdili smo hipotezo, da NTZ pomembno izboljša subjektivno počutje bolnikov, endotelijsko funkcijo in telesno sestavo. NTZ pri naših bolnikih ni izboljšalo metabolične urejenosti.

Literatura

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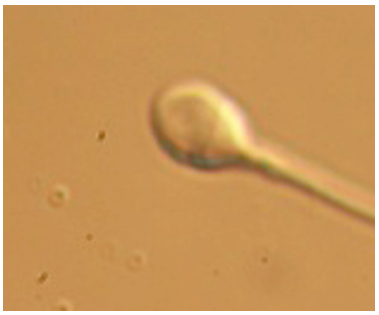
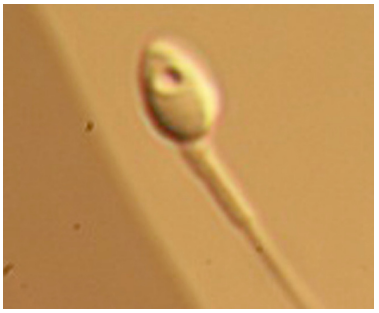
Dodatek - barvne slike

Appendix - Color figures

Slika 1. Spermiji I. razreda I (**A**), II. razreda (**B**) in III. razreda (**Slike C, D** in **E**)



A



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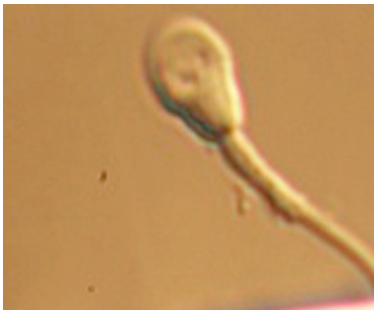

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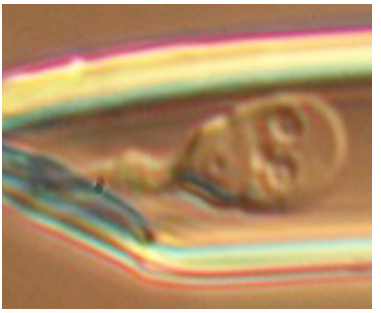
D

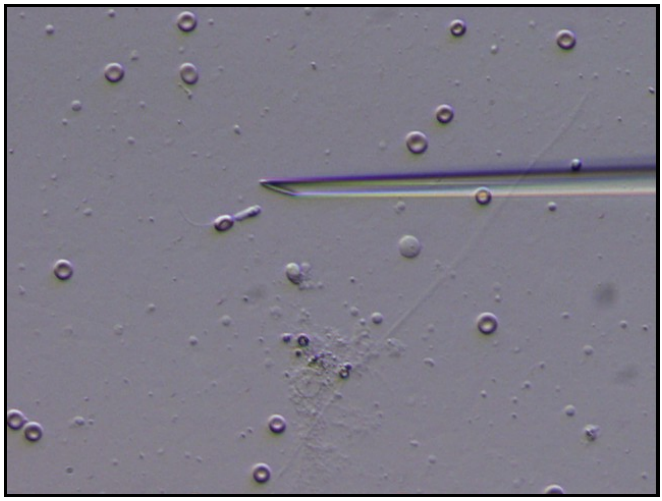


E

Borut Kovačič, Izbira spermija v IVF laboratoriju / Selection of sperm in the IVF laboratory

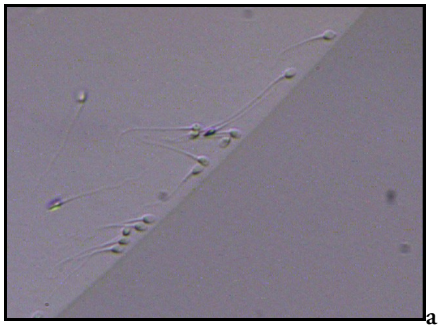
***Slika 1**. "**Swim-up**" laboratorijska metoda za pridobitev gibljivih semenčic iz semenskega izliva moških. Gibljive semenčice plavajo iz sedimenta, v katerem so tudi mrtve celice in levkociti, v višje plasti gojišča. Od tam jih poberemo s pipeto in uporabimo za **osemenitev jajčnih celic in vitro** (a). Za **intracitoplazmatsko injiciranje semenčice** (ICSI) s pipeto osamimo primerno semenčico in jo prenesemo v citoplazmo jajčne celice (b).*

a



b

Slika 2. *Različne metode* ***izbiranja semenčic za ICSI****: Izbor semenčic na robu kapljice polivinilpirolidona, kjer lahko ocenimo njihovo morfologijo (a).* ***Semenčica*** *v suspenziji eritrocitov po* ***aspiraciji moda****. Z dodatkom* ***pentoksifilina*** *povzročimo gibanje bička, s čimer jo lažje izsledimo in potrdimo njeno vitalnost (b). Popolna* ***negibljivost semenčic*** *zaradi nepopolnoma oblikovanih bičkov semenčic (c). Po dodatku* ***hipoozmotskega medija*** *nabreknejo samo repki živih semenčic (d).* ***Levkocitospermija****. Semenčice iščemo na robu kapljice neopranega semena (e).* ***Globozoospermija****. Zaradi odsotnosti akrosoma semenčice kljub metodi ICSI niso sposobne aktivirati in oploditi jajčne celice (f).*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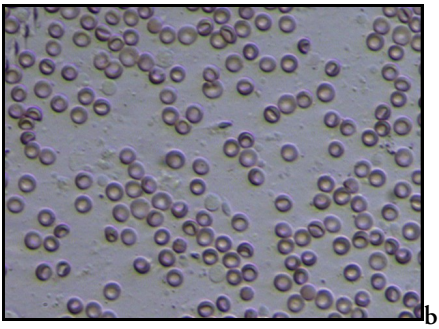
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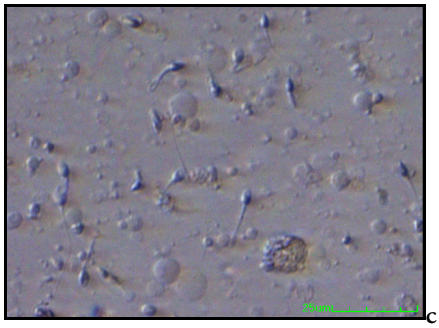
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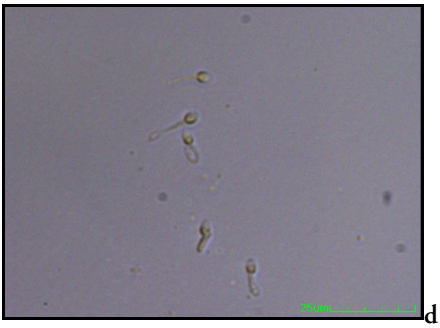
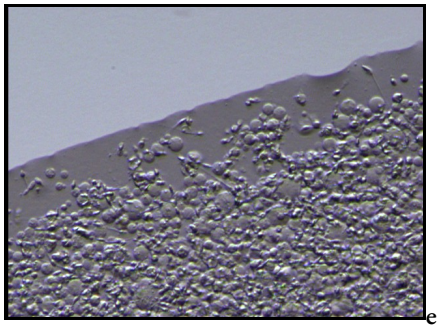

d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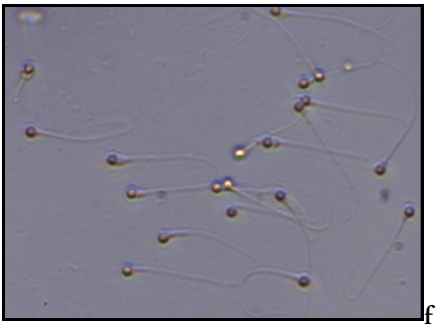
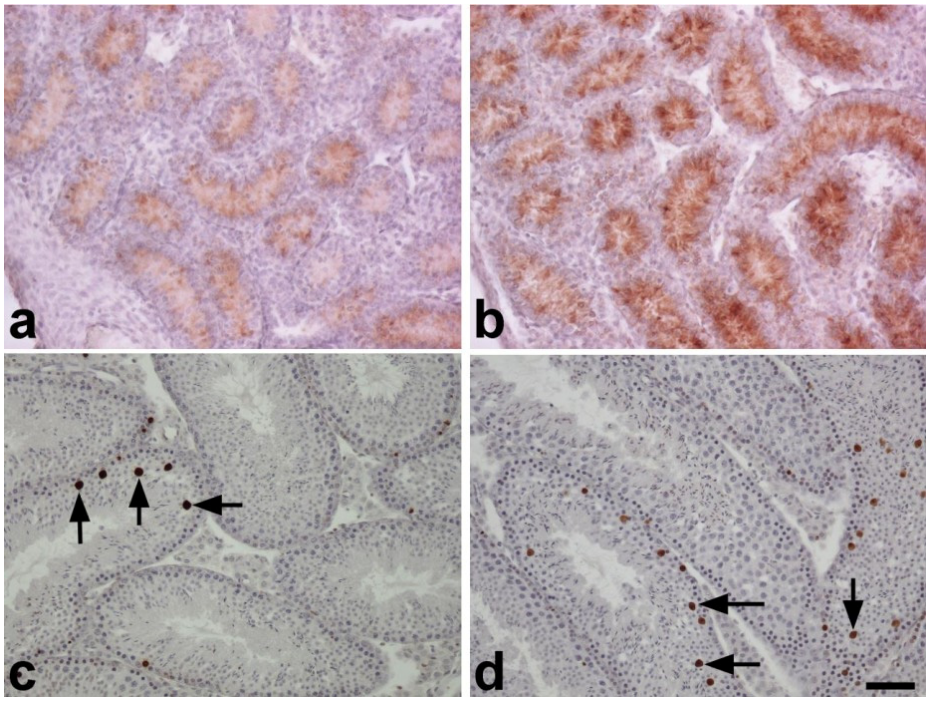

f

Fig. 1. *AMH expression was increased in the testes of the atrazine-exposed group (b) in comparison to the control group (a) on day 9 postnatally. On day 48 postnatally, more apoptotic cells (arrows) were detected in the testes from mice that were neonatally exposed to atrazine (d) in comparison to the control group (c). Bar: 100 µm.*



Sponzorji in pokrovitelji