# Analiza građe spermija prema kriterijima SZO i striktnim kriterijima: usporedba dviju metoda i unutarlaboratorijska varijabilnost

# Sperm morphology assessment according to WHO and strict criteria: method comparison and intra-laboratory variability

Andrea Čipak[1], Patrik Stanić[1], Koraljka Đurić[2], Tihana Serdar[3], Ernest Suchanek[4]

[1]Klinika za ženske bolesti i porode, Klinički bolnički centar "Zagreb", Zagreb
[1]University Department of Obstetrics and Gynecology, Zagreb University Hospital Center, Zagreb, Croatia
[2]Medicinsko biokemijski laboratorij, Poliklinika "Sunce", Zagreb
[2]Medical Biochemistry Laboratory, Sunce Polyclinic, Zagreb, Croatia
[3]Klinički zavod za laboratorijsku dijagnostiku, Klinička bolnica Dubrava, Zagreb
[3]Department of Laboratory Medicine, Dubrava University Hospital, Zagreb, Croatia
[4]Fertility Clinic, Tawam Hospital, Johns Hopkins Medicine, Al Ain, Abu Dhabi, U.A.E.
[4]Fertility Clinic, Tawam Hospital, Johns Hopkins Medicine, Al Ain, Abu Dhabi, U.A.E.

## Sažetak

**Uvod:** Analiza građe spermija jedan je od najvažnijih koraka u procjeni muškog partnera kod neplodnih parova. Međutim, značajne međulaboratorijske i unutarlaboratorijske varijacije mogu uzrokovati poteškoće u tumačenju rezultata, pogrešne dijagnoze te mogu dovesti do zabuna. Stoga je neophodno ove varijacije svesti na najmanju moguću mjeru kako bi se uklonile posljedične greške i osigurala međulaboratorijska i unutarlaboratorijska ponovljivost.

**Materijali i metode:** Uzorci sjemena dobiveni su od 49 uzastopnih muškaraca koji su dolazili u androloški laboratorij radi procjene plodnosti. Usporedili smo dva kriterija za analizu građe spermija: 1) razmaz sjemena pripremljen pomoću boje Giemsa koji smo analizirali prema kriterijima Svjetske zdravstvene organizacije (SZO) i 2) razmaz sjemena pripremljen bojom Spermac koji smo analizirali prema striktnim kriterijima. Također smo proveli i unutarlaboratorijsku usporedbu analize građe spermija.

**Rezultati:** Dijagnosticiranje teratozoospermije prema kriterijima SZO i prema striktnim kriterijima bilo je podudarno kod 45 od 49 ispitanika. Mjera sukladnosti među promatranjima bila je slična i kod kriterija SZO i kod striktnih kriterija (kapa = 0,700 za SZO kriterij; kapa = 0,715 za striktni kriterij).

**Zaključci:** Analize građe spermija prema kriterijima SZO i striktnim kriterijima podudaraju se u postavljanju dijagnoze teratozoospermije i može se postići dobra razina sukladnosti među promatračima nakon odgovarajućeg osposobljavanja, pažljivog pregleda razmaza i poštivanja klasifikacijskih sustava.

**Ključne riječi:** spermiji; neplodnost; muškarac; razlike među ispitivačima

## Abstract

**Background:** Assessment of sperm morphology is one of the most important steps in the evaluation of male partner in infertile couples. However, significant inter- and intra-laboratory variations can cause difficulties in interpretation, misdiagnoses, and consequently lead to confusion. Therefore, it is necessary to minimize these variations to eliminate consequential errors and ensure intra- and inter-laboratory reproducibility.

**Materials and methods:** Semen specimens were obtained from 49 consecutive male patients attending Andrology Laboratory for fertility evaluation. Two sperm morphology assessment criteria were compared: 1) semen smear prepared by Giemsa and assessed by World Health Organization (WHO) criteria; and 2) semen smear prepared by Spermac and assessed by strict criteria. Intra-laboratory comparison of morphological examination was also carried out.

**Results:** The diagnosis of teratozoospermia by both WHO and strict criteria was concordant in 45 of 49 cases. Intra-rater agreement between the observers was similar for WHO and strict criteria (kappa, 0.700 *vs*. 0.715).

**Conclusions:** Morphology assessment by WHO and strict criteria is concordant in diagnosing teratozoospermia and good inter-observer agreement can be achieved after proper training, mindful smear examination and complying with the classification systems.

**Key words:** spermatozoa; infertility; male; observer variation

*Pristiglo: 23. listopada 2008.* *Received: October 23, 2008*
*Prihvaćeno: 8. prosinca 2008.* *Accepted: December 8, 2008*

## Uvod

Analiza građe spermija, sastavnica analize sjemena, jedan je od najvažnijih koraka u procjeni muškog partnera kod neplodnih parova. Svjetska zdravstvena organizacija (SZO) dosada je objavila nekoliko priručnika s ciljem standardizacije postupaka analize sjemena te su kriteriji SZO postali najšire prihvaćeni u pretragama građe spermija u androloškim laboratorijima diljem svijeta (1,2). Nadalje, Menkveld i sur. su 1990. godine pokazali da analiza građe spermija prema strožim kriterijima, takozvanim Tygerbergovim ili striktnim kriterijima, povećava objektivnost i smanjuje unutarlaboratorijsku varijabilnost (3). Međutim, zbog različitih klasifikacijskih sustava, značajne među- i unutarlaboratorijske varijacije i dalje su prisutne zbog mnoštva čimbenika, kao što su tehnike pripreme razmaza, tumačenje rezultata i iskustvo laboratorijskih tehničara (4). Takve varijacije mogu uzrokovati poteškoće kod tumačenja rezultata, pogrešnu dijagnozu te naposljetku dovesti do zabuna. Stoga ih je neophodno svesti na najmanju moguću mjeru, radi uklanjanja posljedičnih pogrešaka i osiguranja unutar- i međulaboratorijske ponovljivosti.

Cilj ovoga istraživanja bio je usporediti dva kriterija analize građe spermija: 1) razmaz sjemena pripremljen pomoću boje Giemsa i analiziran prema kriterijima SZO te 2) razmaz sjemena pripremljen pomoću boje Spermac i analiziran prema striktnim kriterijima. Također smo željeli provesti unutarlaboratorijsku usporedbu analize građe spermija. Prema našim saznanjima, ovo je prvo takvo izvješće usporedbe metoda i unutarlaboratorijskih varijacija provedeno u Hrvatskoj.

## Materijali i metode

### Ispitanici

Uzorci sjemena dobiveni su od 49 uzastopnih muškaraca uključenih u istraživanje, životne dobi između 18 i 50 godina. Ispitanici su dolazili u Androloški laboratorij Klinike za ženske bolesti i porode, Kliničkog bolničkog centra „Zagreb" u Zagrebu, radi procjene plodnosti, u razdoblju od svibnja do srpnja 2007. Svi su sudionici prije analize najmanje 2 dana apstinirali od spolnih odnosa, odnosno ejakulacije.

### Kriteriji

Prema kriterijima SZO (1) normozoospermija definira ejakulat s koncentracijom spermija većom od 20 x $10^6$ spermija/mL, progresivne pokretljivosti spermija > 50% ili barem 25% spermija s linearno progresivnom pokretljivošću i ≥ 30% spermija s normalnom građom. Međutim, ovi se kriteriji mogu koristiti samo kada se analiza sjemena izvodi na 37 °C. Temperatura jako utječe na pokretljivost spermija te bi se ona uvijek trebala analizirati pri kontro-

## Introduction

Assessment of sperm morphology as a component of semen analysis is one of the most important steps in the evaluation of male partner in infertile couples. The World Health Organization (WHO) has so far published several manuals in order to standardize semen analysis procedures and WHO criteria have become widely accepted in sperm morphology examination at andrology laboratories all over the world (1,2). Furthermore, in 1990, Menkveld *et al*. showed that the assessment of sperm morphology by more stringent criteria, the so called Tygerberg or strict criteria enhances objectivity and decreases intra-laboratory variability (3). Nevertheless, due to different classification systems, a significant inter- and intra-laboratory variation also exists as a result of many factors including different smear preparation techniques, interpretation and technician experience (4). Such variations can cause difficulties in interpretation, misdiagnoses, and consequently lead to confusion. Therefore, it is necessary to minimize these variations to eliminate consequential errors and ensure intra- and inter-laboratory reproducibility.

The aim of our study was to compare two sperm morphology assessment criteria: 1) semen smear prepared by Giemsa and assessed by WHO criteria; and 2) semen smear prepared by Spermac and assessed by strict criteria. We also aimed to carry out intra-laboratory comparison of morphological examination. To the best of our knowledge, this is the first report on comparison of methods and intra-laboratory variations in Croatia.

## Materials and methods

### Patients

Semen specimens were obtained from 49 consecutive male patients between 18 and 50 years of age. The patients attended Andrology Laboratory, University Department of Obstetrics and Gynecology, Zagreb University Hospital Center, for fertility evaluation in the period from May to July 2007. All subjects were asked for a minimum of 2 days of sexual abstinence.

### Criteria

According to WHO criteria (1), normozoospermia is defined as an ejaculate with sperm concentration of > 20 x $10^6$ spermatozoa/mL, progressive sperm motility of > 50%, or at least 25% of spermatozoa with linear progressive motility and ≥ 30% of morphologically normal spermatozoa. However, these criteria can only be applied when sperm analysis is carried out at 37 °C. Sperm motility is highly influenced by temperature and should always be assessed under controlled thermal conditions (5). Since, the motility analysis at Andrology Laboratory was performed at room temperature (22 °C), we had to

liranim temperaturnim uvjetima (5). Budući da se analiza pokretljivosti spermija u Androloškom laboratoriju Klinike za ženske bolesti i porode izvodila na sobnoj temperaturi (22 °C), morali smo prilagoditi kriterije pokretljivosti spermija. Prema tim prilagođenim kriterijima, astenozoospermija je prisutna ukoliko je nađeno < 40% spermija s progresivnom pokretljivošću u uzorku sjemena (5). Teratozoospermija dijagnosticira ejakulat s < 30% normalno građenih spermija prema kriterijima SZO ili < 15% prema striktnim kriterijima. Oligozoospermija definira ejakulat s koncentracijom spermija < 20 x $10^6$ spermija/mL. Naposljetku, oligoastenozoospermija dijagnosticira ejakulat sa smanjenom koncentracijom i pokretljivošću spermija.

**Analiza pokretljivosti i koncentracije spermija**

Pokretljivost i koncentracija spermija određivana je upotrebom Autosperm, Amsaten Corp. (De Pinte, Belgija) sustava za analizu ejakulata. Nakon likvefakcije 10 µL sjemena je pipetirano na predmetno stakalce i pokriveno pokrovnicom (veličina 22 x 22 mm). Analiza se izvodila na sobnoj temperaturi pri povećanju od 500 puta. Pokretljivost je izražena kao postotak pokretnih spermija:

a) spermiji s linearnom i progresivnom pokretljivošću (linearna brzina ≥ 22 µm/s);
b) spermiji sa sporom linearnom ili nelinearnom pokretljivošću (linearna brzina < 22 µm/s i brzina ≥ 5 µm/s);
c) na mjestu pokretni spermiji; i
d) nepokretni spermiji.

**Analiza građe spermija prema kriterijima SZO**

Nakon likvefakcije, napravio se razmaz od 10 µL sjemena na predmetnom stakalcu i ostavio na sobnoj temperaturi da se osuši. Razmazi su tada obojani bojom Giemsa i građa spermija je određivana prema kriterijima SZO (1). Dva su različita promatrača prebrojala 200 stanica po razmazu, koristeći svjetlosnu mikroskopiju pri povećanju od 1000 puta i uljnu imerziju. Prema kriterijima SZO normalno građen spermij ima glavu ovalnog oblika i akrosom koji pokriva 40–70% područja glave. Normalni spermij nema nepravilnosti u vratu, srednjem dijelu i repu, niti ostatna tjelešca veća od 50% veličine glave.

**Kvantifikacija leukocita i stanica spermatogenske loze**

Leukociti i stanice spermatogenetske loze (okrugle spermatide, spermatociti i spermatogonije) analizirani su bojanjem predmetnih stakalaca bojom Giemsa i kvantificirani Autosperm, Amsaten Corp. (De Pinte, Belgija) sustavom za analizu ejakulata.

**Analiza građe spermija prema striktnim kriterijima**

Nakon likvefakcije, a prije bojanja alikvot sjemena ispran je sredstvom Quinn's Sperm Washing Medium, SAGE (SAD) i centrifugiran 10 minuta na 300 g. Odvojen je supernatant, a talogu je dodano 0,5 mL Quinn sredstva za

readjust the sperm motility criteria. According to these readjusted criteria, asthenozoospermia is present when < 40% of spermatozoa with progressive motility is found in the semen sample (5). Teratozoospermia is diagnosed when < 30% of morphologically normal spermatozoa are found in semen samples according to the WHO criteria or < 15% according to the strict criteria. Oligozoospermia defines an ejaculate with sperm concentration of < 20 x $10^6$ spermatozoa/mL. Finally, oligoasthenozoospermia is defined as an ejaculate with reduced sperm concentration and motility.

**Assessment of sperm motility and concentration**

The evaluation of sperm motility and concentration was performed by using the Autosperm, Amsaten Corp. (De Pinte, Belgium) system for ejaculate analysis. After liquefaction, 10 µL of semen was pipetted onto a glass slide and covered with a cover slip (size 22 x 22 mm). The analysis was performed at room temperature at final magnification of 500x. The motility was expressed as the percentage of motile spermatozoa:

a) spermatozoa with linear and progressive motility (linear velocity ≥ 22 µm/s);
b) spermatozoa with slow linear or nonlinear motility (linear velocity < 22 µm/s and velocity ≥ 5 µm/s);
c) sluggish; and
d) immotile spermatozoa.

**Sperm morphology assessment by WHO criteria**

Following liquefaction, 10 µL of semen was spread onto a glass slide and allowed to air-dry at room temperature. The smears were then stained with Giemsa stain and sperm morphology was assessed according to WHO criteria (1). Two different examiners counted 200 cells *per* smear using brightfield illumination at final magnification of 1000x and oil immersion. According to WHO criteria, a morphologically normal spermatozoon has an oval head and an acrosome covering 40%–70% of the head area. A normal spermatozoon has no neck, midpiece, tail abnormalities nor cytoplasmic droplets larger than 50% of the sperm head.

**Quantification of leukocytes and immature germ cells**

Leukocytes and immature germ cells (round spermatids, spermatocytes and spermatogonia) were assessed by staining the slides with Giemsa stain and quantified by using the Autosperm, Amsaten Corp. (De Pinte, Belgium) system for ejaculate analysis.

**Sperm morphology assessment by strict criteria**

After liquefaction and prior to staining, an aliquot of semen was washed with Quinn's Sperm Washing Medium, SAGE (USA) and centrifuged at 300 g for 10 minutes. The supernatant was removed and 0.5 mL of Quinn's me-

ispiranje spermija. Napravljen je razmaz od 10 µL ispranog sjemena na predmetnom stakalcu koji se zatim fiksirao i sušio na zraku. Razmazi su tada isprani destiliranom vodom i obojani bojom Spermac, FertiPro (Beernem, Belgija). Nakon bojanja razmazi su isprani destiliranom vodom. Dva su različita promatrača brojala 200 stanica po razmazu koristeći se mikroskopom s povećanjem od 1000 puta i uljnom imerzijom. Za procjenu građe korišteni su striktni kriteriji prema kojima je spermij normalno građen ako ima ovalnu glavu duljine 4,0–5,0 µm i širine 2,5–3,5 µm, što se mjerilo okularnim mikrometrom. Omjer duljine prema širini trebao bi iznositi 1,50–1,75. Normalni spermij ima dobro izražen akrosom, koji pokriva 40–70% glave. Srednji dio je tanak, tanji od 1 µm, i otprilike 1,5 puta dulji od glave. Ostatna tjelešca, ukoliko su prisutna, ne bi trebala biti veća od polovine širine glave. Rep je tanak, uniforman, nesavijen i otprilike 45 µm dugačak. Prema ovom klasifikacijskom sustavu svi se granični oblici smatraju nepravilnima (3).

**Indeks teratozoospermije**

Indeks teratozoospermije (engl. *teratozoospermia index*, TZI) definira se kao broj anomalija po nepravilnom spermiju. Svi spermiji nepravilnog oblika mogu imati jednu do četiri nepravilnosti, uključujući anomalije glave, vrata/srednjeg dijela i repa ili prisutnost ostatnih tjelešaca (1). Klasifikacija spermija za TZI izvodi se na laboratorijskom brojaču. Spermiji se klasificiraju kao normalni i abnormalni te se svrstavaju u specifične skupine (nepravilnosti glave, vrata/srednjeg dijela i repa ili skupina ostatnih tjelešaca). Ukupne vrijednosti pronađenih nepravilnosti se tada zbroje i podijele s brojem nepravilnih spermija.

**Test vitalnosti pomoću eozina**

Vitalnost se utvrđivala bojenjem razmaza pomoću eozina (1). Vitalni spermiji imaju intaktnu staničnu membranu, ne boje se te ostaju bijeli u razmazu, dok eozin prodire kroz oštećenu staničnu membranu spermija koji nisu vitalni. Razmazi su ostavljeni da se osuše na zraku, a procjena je izvršena brojanjem crveno obojanih i neobojanih spermija pomoću svjetlosne mikroskopije kod povećanja od 400 puta. Izbrojeno je najmanje 100 stanica. Rezultati su prikazani kao postotak eozin negativnih (neobojanih) spermija.

**Statistička analiza**

Rezultati su opisani aritmetičkom sredinom i standardnom devijacijom. Sve je uzorke sjemena analizirao jedan ocjenjivač. Razlike između parametara sjemena analiziranih prema kiterijima SZO i prema striktnim kriterijima ispitane su t-testom. Razlike u parametrima sjemena među različitim skupinama (normozoospermija, astenozoospermija, oligozoospermija i oligoastenozoospermija) analizirane su jednosmjernom analizom varijance ANOVA. Ocijenjena je i mjera usklađenosti promatrača za oba kriteridium was added to the remaining pellet. Ten µL of washed semen was then spread onto a glass slide, fixed and air-dried. The smears were washed with distilled water and stained with Spermac stain, FertiPro (Beernem, Belgium). After staining, the smears were washed with distilled water. Two different examiners counted 200 cells *per* smear using brightfield illumination at final magnification of 1000x and oil immersion. Strict criteria (3) were applied for the evaluation, according to which a spermatozoon is normal if it has an oval head, 4.0–5.0 µm long and 2.5–3.5 µm wide, measured with an ocular micrometer. The length-to-width ratio should be 1.50–1.75. A normal spermatozoon has a well-defined acrosome that covers 40%–70% of the head. The midpiece is thin, less than 1 µm wide, about 1.5 times longer than the head. Cytoplasmic droplets, if present, should not be larger than half of the head width. The tail is thin, uniform, uncoiled and about 45 µm long. According to this classification system, all borderline forms are considered as abnormal (3).

**Teratozoospermia index**

Teratozoospermia index (TZI) is defined as the number of abnormalities present *per* abnormal spermatozoon. All abnormal spermatozoa can have one to four abnormalities, including head, neck/midpiece and tail defects or presence of cytoplasmic droplets (1). The classification of spermatozoa for TZI is carried out on a laboratory counter. The spermatozoa are recorded as normal or abnormal and distributed into specific groups (head, neck/midpiece and tail defects or cytoplasmic droplet groups). The total number of abnormalities is then added together and divided by the number of abnormal spermatozoa.

**Eosin test**

The vitality was analyzed by staining the smears with eosin (1). Vital sperms have intact cell membrane, are not stained and remain white in the smear, whereas eosin diffuses through the disrupted cell membrane of the sperms which are not vital. The smears were air-dried and the evaluation was performed by counting the red-stained and unstained spermatozoa with brightfield optics at final magnification of 400x. At least 100 cells were counted. Results are presented as proportion (%) of eosin negative (unstained) sperms.

**Statistical analysis**

The results were described with arithmetic mean and standard deviation. All semen samples were assessed by one observer. Differences between semen parameters assessed by WHO and strict criteria were tested by t-test. Differences in semen parameters among various subgroups (normozoospermia, asthenozoospermia, oligozoospermia and oligoasthenozoospermia) were assessed by one-way ANOVA. Inter-observer agreement was examined on the evaluation of the sperm morphology by

rija analize građe spermija te izračunata njena vrijednost, kapa. Rezultati su analizirani programom SSPS, SPSS Inc. (Chicago, SAD).

## Rezultati

U Tablici 1. prikazana su svojstva sjemena, zajedno s dobi ispitanika i vremenom apstinencije. Ispitanici su raspoređeni u četiri skupine: skupina s dijagnozom normozoospermije, astenozoospermije, oligozoospermije i oligoastenozoospermije. Nije nađena statistički značajna razlika u dobi i duljini apstinencije između te četiri skupine. Međutim, nađena je statistički značajna razlika između skupina u koncentraciji spermija, ukupnom broju spermija, njihovoj pokretljivosti i vitalnosti (P < 0,001 za svaki od parametara). Također su se volumen sjemena te broj stanica spermatogenetske loze u sjemenu statistički značajno razlikovali između skupina (P = 0,029 i 0,024).

Rezultati analize građe spermija i TZI prikazani su u Tablici 2. Spermiji normalne građe bili su prisutni kod 18 od 49 muškaraca prema kriterijima SZO i kod 16 od 49 prema striktnim kriterijima. Kriteriji SZO i striktni kriteriji su bili podudarni u 45 od 49 slučajeva u dijagnosticiranju teratozoospermije.

U ispitanika s normozoospermijom, astenozoospermijom i oligoastenozoospermijom nađeno je statistički značajno više nepravilnosti glave spermija (P = 0,001, 0,020 i 0,031), kada je građa spermija određivana prema kriterijima SZO u odnosu prema striktnim kriterijima. U skupini s oligoastenozoospermijom nađeno je statistički značajno više nepravilnosti u području vrata i srednjeg dijela spermija (P = 0,005) dok je u skupinama s normozoospermijom i astenozoospermijom nađeno statistički značajno više nepravilnosti repa spermija (P = 0,002 i 0,005) kada je

both WHO and strict criteria. Kappa value was calculated as a measure of inter-observer agreement. The results were analyzed with the SPSS program, SPSS Inc. (Chicago, USA).

## Results

Semen parameters, together with patient age and sexual abstinence are presented in Table 1. The patients were distributed into 4 groups as follows: normozoospermia, asthenozoospermia, oligozoospermia and oligoasthenozoospermia. No statistically significant difference was found in the age or sexual abstinence among the four groups. However, there was a considerable inter-group difference in sperm concentration, total sperm count, motility and vitality (P < 0.001 for each parameter). Also, the semen volume and the number of immature germ cells differed significantly between the groups (P = 0.029 and 0.024, respectively).

The results of sperm morphology assessment and TZI are presented in Table 2. Normal sperm morphology was present in 18 of 49 men by WHO criteria and in 16 of 49 men by strict criteria. The diagnosis of teratozoospermia by both WHO and strict criteria were concordant in 45 of 49 cases.

The mean percentage of sperm head defects was markedly higher in the normozoospermia, asthenozoospermia and oligoasthenozoospermia groups when sperm morphology was assessed by WHO criteria compared to strict criteria (P = 0.001, 0.020 and 0.031, respectively). The mean percentage of sperm neck and midpiece defects in the oligoasthenozoospermia group (P = 0.005) and the mean percentage of sperm tail defects in normozoospermia and asthenozoospermia groups were significantly

**Tablica 1.** Ispitanici i svojstva sjemena

**Table 1.** Patient and semen parameters

| Patients | N (N = 15) | A (N = 13) | O (N = 7) | OA (N = 14) | P |
|---|---|---|---|---|---|
| Age (years) | 34 ± 6 | 33 ± 6 | 37 ± 6 | 33 ± 7 | 0.413 |
| Sexual abstinence (days) | 4 ± 1 | 4 ± 2 | 2 ± 1 | 4 ± 2 | 0.258 |
| Semen volume (mL) | 4.07 ± 1.25 | 3.73 ± 1.09 | 2.24 ± 0.82 | 3.22 ± 1.77 | **0.029** |
| Semen pH | 7.90 ± 0.21 | 7.92 ± 0.24 | 8.11 ± 0.38 | 7.97 ± 0.31 | 0.380 |
| Immature germ cells (x $10^6$/mL) | 2.36 ± 1.40 | 2.00 ± 2.30 | 0.71 ± 0.82 | 0.85 ± 0.80 | **0.024** |
| Leukocytes in semen (x $10^6$/mL) | 0.16 ± 0.13 | 0.16 ± 0.26 | 0.61 ± 1.36 | 0.40 ± 0.57 | 0.316 |
| Sperm concentration (x $10^6$/mL) | 67.60 ± 28.11 | 35.04 ± 15.80 | 14.11 ± 4.45 | 9.56 ± 4.69 | **<0.001** |
| Total sperm count (x $10^6$) | 268.92 ± 134.37 | 130.70 ± 63.74 | 32.47 ± 17.23 | 30.91 ± 21.57 | **<0.001** |
| Sperm motility (grade a+b) (%) | 53.60 ± 6.51 | 21.53 ± 11.70 | 49.42 ± 8.69 | 17.78 ± 10.78 | **<0.001** |
| Sperm vitality (%) | 76.40 ± 4.74 | 53.23 ± 18.58 | 70.00 ± 5.03 | 46.42 ± 16.49 | **<0.001** |

N – normozoospermia; A – asthenozoospermia; O – oligozoospermia; OA – oligoasthenozoospermia

**Tablica 2.** Rezultati analize građe spermija

**Table 2.** Results of sperm morphology assessment

| Patients | | N (N = 15) | | | A (N = 13) | | | O (N = 7) | | | OA (N = 14) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | WHO | SC | P | WHO | SC | P | WHO | SC | P | WHO | SC | P |
| **Normal morphology (%)** | | 29 ± 8 | 16 ± 6 | **0.001** | 24 ± 9 | 11 ± 4 | **0.001** | 26 ± 9 | 14 ± 73 | **0.018** | 13 ± 7 | 6 ± 4 | **0.001** |
| **Categories of sperm abnormality in semen samples** | **Head abnormalities (%)** | 74 ± 4 | 71 ± 4 | **0.001** | 68 ± 4 | 66 ± 4 | **0.020** | 69 ± 2 | 66 ± 4 | 0.088 | 65 ± 7 | 63 ± 7 | **0.031** |
| | **Neck and midpiece abnormalities (%)** | 12 ± 3 | 12 ± 4 | 0.551 | 15 ± 3 | 15 ± 3 | 0.964 | 14 ± 2 | 15 ± 2 | 0.752 | 14 ± 3 | 16 ± 3 | **0.005** |
| | **Tail abnormalities (%)** | 12 ± 3 | 15 ± 2 | **0.002** | 15 ± 3 | 18 ± 3 | **0.005** | 15 ± 2 | 17 ± 4 | 0.236 | 19 ± 4 | 19 ± 4 | 0.813 |
| | **Cytoplasmatic droplets (%)** | 2 ± 1 | 2 ± 1 | 1.000 | 2 ± 1 | 1 ± 1 | 0.527 | 2 ± 2 | 2 ± 1 | 0.655 | 2 ± 2 | 2 ± 1 | 0.132 |
| | **Teratozoospermia index** | 1.16 ± 0.42 | 1.27 ± 0.32 | **0.040** | 1.44 ± 0.09 | 1.47 ± 0.09 | 0.278 | 1.27 ± 0.81 | 1 46 ± 0.07 | 0.352 | 1.50 ± 0.13 | 1.46 ± 0.42 | 0.451 |

WHO – WHO criteria; SC – strict criteria.
N – normozoospermia; A – asthenozoospermia; O – oligozoospermia; OA – oligoasthenozoospermia

građa spermija određivana prema striktnim kriterijima u odnosu prema kriterijima SZO. TZI je bio statistički značajno viši (P = 0,040) u skupini s normozoospermijom kada se građa spermija određivala prema striktnim kriterijima u odnosu prema standardima SZO.
Primijetili smo visok stupanj podudaranja između promatrača kod oba kriterija za analizu građe spermija. Mjera sukladnosti bila je slična kod kriterija SZO i striktnih kriterija (kapa = 0,700 za SZO prema 0,715 za striktne kriterije).

## Rasprava

Ključni rezultat ovoga istraživanja bio je da je usporedba analize građe spermija prema kriterijima SZO i striktnim kriterijima pokazala maksimalnu podudarnost u dijagnosticiranju teratozoospermije. Također, unutarlaboratorijska usporedba je pokazala dobru mjeru sukladnosti za oba kriterija procjene građe spermija.
Mnogi su autori istraživali utjecaj tehnika pripreme i kriterija procjene na objektivnost analize građe spermija i unutarlaboratorijsku varijabilnost. Međutim, zbog različito ustrojenih istraživanja nije jednostavno usporediti rezultate dosada objavljene u literaturi. Godine 1993. Meschede i sur. su istraživali utjecaj triju različitih tehnika pripreme razmaza: Papanicolau, Shorr bojanje i protokol „*wet preparations*" na rezultate analize građe spermija (6). To je istraživanje pokazalo vrlo slabu povezanost između

higher when sperm morphology was assessed by strict criteria compared to WHO criteria (P = 0.002 and 0.005, respectively). TZI was markedly higher (P = 0.040) in the normozoospermia group when sperm morphology was assessed by strict criteria compared to WHO criteria.
We observed a good level of agreement between the observers for both sperm morphology assessment criteria. Intra-rater agreement was similar for WHO and strict criteria (kappa, 0.700 *vs*. 0.715).

## Discussion

The key finding of our study was that comparison of sperm morphology assessment by WHO and strict criteria showed maximal concordance in diagnosing teratozoospermia. Also, a good inter-observer agreement for both criteria was found in the intra-laboratory comparison of morphology assessment.
Many authors have studied the impact of different preparation techniques and assessment criteria on the objectivity of morphology assessment and intra-laboratory variability. However, due to different study designs it is quite uneasy to compare results so far published in the literature. In 1993, Meschede *et al*. studied the influence of three different preparation techniques, i.e. Papanicolaou, Shorr stain and 'wet preparations' protocol, on the results of sperm morphology assessment (6). This study showed

tehnika i stoga su preporučili da se u jednom laboratoriju upotrebljava samo jedna metoda kako bi se osigurala usporedivost laboratorija. Međutim, drugi su istraživači (7,8) pronašli dobru povezanost između metoda bojanja koje su rabili u svojim istraživanjima. Ova nepodudarnost objavljenih rezultata mogla bi biti posljedicom značajnih poboljšanja koja su postignuta u standardizaciji analize građe spermija kao i poboljšanja u kontroli kvalitete analize sjemena postignutih tijekom posljednjih 15 godina. Rezultati našega istraživanja sukladni su nedavno objavljenim radovima te dokazuju da se prihvatljivo podudaranje može postići kada se klasifikacijski sustavi prilagode tehnikama bojanja.

Bojanje bojom Giemsa koje se rutinski rabi u našem laboratoriju dalo je povećan broj nepravilnosti glave spermija kod skupina s normozoospermijom, astenozoospermijom i oligoastenozoospermijom kada se građa spermija određivala prema kriterijima SZO, u odnosu prema striktnim kriterijima. To bi moglo biti zbog obojenosti pozadine neispranih uzoraka i slične obojenosti različitih dijelova spermija. S druge strane, kada su uzorci bili obojani bojom Spermac, različiti dijelovi spermija su bili različito obojani, što je rezultiralo boljim vizualnim zapažanjem svih nepravilnosti spermija.

Nadalje, metoda pripreme može značajno utjecati i na TZI (6). Međutim, u našem istraživanju metoda pripreme nije utjecala na TZI. Kako je navedeno, usporedba analize građe spermija prema oba kriterija bila je podudarna u dijagnosticiranju teratozoospermije, dok je najveća nepodudarnost pri određivanju TZI bila kod skupine s normozoospermijom.

Što se naših rezultata tiče, unutarlaboratorijska usporedba otkrila je dobar stupanj mjere sukladnosti između dvoje promatrača u našem laboratoriju. Međutim, u jednom istraživanju provedenom na 54 uzoraka sjemena 8 plodnih muškaraca i 46 muškaraca smanjene plodnosti u kojem su se rabili likveficirani i oprani uzorci obojeni bojama Diff-Quik i Papanicolau primijećen je visok stupanj mjere sukladnosti između dvoje promatrača (međupromatračka varijabilnost za likveficiran i opran uzorak iznosila je 0,82 za Diff-Quick i 0,93 za Papanicolau) (9). Od to dvoje promatrača jedan je bio iskusan znanstvenik s više od 5 godina iskustva, dok je drugi bio znanstvenik s manje od dvije godine iskustva. Praktično iskustvo i poštivanje preporučenih metodologija od ključnog su značenja u dijagnosticiranju teratozoospermije i mogu imati velik utjecaj na rezultate analize građe spermija (10). Jedan od naših promatrača se tek nedavno upoznao s analizom građe spermija. To bi mogao biti razlog nešto nižeg stupnja mjere sukladnosti. No bez obzira na to, ovaj nam slučaj pruža dobar primjer kako se nakon odgovarajućeg osposobljavanja, pažljive pripreme razmaza i poštivanja klasifikacijskih sustava mogu postići rezultati slični onima iskusnog promatrača. Mnogi su autori (3,4,10,13) proveli usporedbe

very poor correlation among the techniques; therefore the authors recommended that only one method be used in a particular laboratory in order to ensure laboratory comparability. However, other researches (7,8) found good correlation between staining methods used in their study. The inconsistency in observed in the reported results might be due to the significant improvements made in sperm morphology assessment standardization as well as improvements in quality control of semen analysis in the last 15 years. The results of our study are in close agreement with latest reports, proving that acceptable agreement can be accomplished when classification systems are adjusted to the staining techniques.

Giemsa staining, routinely used in our laboratory, yielded a higher mean percentage of head abnormalities in the normozoospermia, asthenozoospermia and oligoasthenozoospermia groups when morphology was assessed by WHO criteria compared to strict criteria. This might be due to the background staining of unwashed samples and similar staining of different sperm parts. On the other hand, when samples were stained by Spermac, various sperm parts were stained with different colors, resulting in better visual perception of all sperm abnormalities.

Moreover, TZI may also be significantly influenced by the preparation method (6). On the contrary, TZI was not influenced by the preparation method in our study. As already stated, comparison of sperm morphology assessment by both criteria was most consistent in diagnosing teratozoospermia, whereas the major inconsistency in observing TZI was found in the normozoospermia group.

As of our results, the intra-laboratory comparison revealed a good level of agreement between the two observers in our laboratory. However, in one study on 54 semen samples from eight fertile men and 46 subfertile patients using liquefied and washed samples stained with Diff-Quik and Papanicolaou stains, a very high degree of agreement (inter-observer variability for liquefied and washed samples of 0.82 and 0.93, respectively) was observed between the two observers (9). Of those two examiners, one was a trained scientist with more than 5-year experience, while the other one was a scientist with less than 2-year experience. Practical experience and compliance with the recommended methodologies are crucial in diagnosing teratozoospermia and can have a huge impact on the results of the sperm morphology assessment (10). One of our observers was newly introduced to the sperm morphology assessment. This could be the reason for a somewhat lesser degree of inter-rater agreement. Even so, this provides a good example of how, after proper training, mindful smear examination and complying with the classification systems, results similar to those obtained by an experienced observer could be achieved. Many authors (3,4,10,13) carried out intra-/inter-observer and/or intra-/inter-laboratory comparisons. Unfortunate-

rezultata između više promatrača/jednog promatrača i/ili međulaboratorijske/unutarlaboratorijske usporedbe. Nažalost, prilično je kompliciranо uspoređivati njihove rezultate zbog velikih razlika u ustrojstvima istraživanja i mjerama varijabilnosti.

Temeljem naših rezultata možemo zaključiti da su analize građe spermija prema kriterijima SZO i striktnim kriterijima podudarne u postavljanju dijagnoze teratozoospermije, bez obzira na primijenjene kriterije, i da se dobra mjera sukladnosti može postići ako se prije procjene građe uzmu u obzir sve neophodne mjere predostrožnosti dobre laboratorijske prakse.

Građa spermija prihvaćena je kao parametar sjemena koji najviše korelira sa sposobnošću oplodnje *in vivo* (11) i *in vitro* (12). Međutim, u tijeku je rasprava o pouzdanosti rezultata analize sjemena (13). To tek ukazuje na potrebu za standardizacijom i kontinuiranim praćenjem kvalitete. Nadamo se da će ovo istraživanje potaknuti ostale androloške laboratorije u Hrvatskoj na analizu svoje mjere usklađenosti. To bi mogla biti dobra inicijativa za standardizaciju analize građe spermija na razini Republike Hrvatske.

**Zahvala**

Ovo je istraživanje provedeno uz potporu Ministarstva znanosti, obrazovanja i športa Republike Hrvatske. Broj projekta: 108-1080399-0384.

ly, it is quite difficult to compare their results, due to some major differences in study design and measures of variability.

Based on our results herein we conclude that morphology assessment by WHO and strict criteria is concordant in diagnosing teratozoospermia regardless of the criteria used and that a good inter-observer agreement can be achieved when all necessary precautions of good laboratory practice are taken into consideration prior to morphology evaluation.

Sperm morphology is recognized as a semen parameter that mostly correlates with the *in vivo* (11) and *in vitro* fertilizing ability (12). Nevertheless, there is an ongoing debate regarding the reliability of the results of semen analysis (13). It points to the need of standardization and continuous quality monitoring. Hopefully, this study will encourage other Croatian andrology laboratories to investigate their inter-rater agreement. This could be a good initiative for standardization of sperm morphology assessment at the national level.

**Acknowledgments**

This work was supported by the Ministry of Science, Education and Sports, Republic of Croatia, project number 108-1080399-0384.

**Adresa za dopisivanje:**

Andrea Čipak
Klinički zavod za kemiju
Klinička bolnica "Sestre milosrdnice"
Vinogradska cesta 29
10000 Zagreb
e-pošta: andrea.cipak@gmail.com

**Corresponding author:**

Andrea Čipak
University Department of Chemistry
Sestre milosrdnice University Hospital
Vinogradska cesta 29
HR-10000 Zagreb
Croatia
e-mail: andrea.cipak@gmail.com

**Literatura/References**

1. *World Health Organization. WHO Laboratory Manual for the Examination of Human Semen and Semen-Cervical Mucus Interaction. 3rd edition. Cambridge: Cambridge University Press; 1992.*
2. *World Health Organization. WHO Laboratory Manual for the Examination of Human Semen and Semen-Cervical Mucus Interaction. 4th edition. Cambridge: Cambridge University Press; 1999.*
3. *Menkveld R, Stander FSH, Kotze TJVW, Kruger TF, van Zyl JA. The evaluation of morphological characteristics of human spermatozoa according to stricter criteria. Hum Reprod 1990;5:586-92.*
4. *Coetzee K, Kruger TF, Lombard CJ, Shaughnessy D, Oehringer S, Özgür K, et al. Assessment of interlaboratory and intralaboratory sperm morphology readings with the use of a Hamilton Thorne Research integrated visual optical system semen analyzer. Fertil Steril 1999;71:80-4.*
5. *Jeyendran RS. Protocols for Semen Analysis in Clinical Diagnosis. London: Parthenon Publishing Group; 2003. pp. 31-32.*
6. *Meschede D, Keck C, Zander M, Cooper TG, Yeung CH, Nieschlag E. Influence of three different preparation techniques on the results of human sperm morphology analysis. Int J Androl 1993;16:362-9.*
7. *Menkveld R, Lacquet FA, Kruger TF, Lombard CJ, Sanchez Sarmiento CA, de Villiers A. Effects of different staining and washing procedures on the results of human sperm morphology evaluation by manual and computerised methods. Andrologia 1997;29:1-7.*
8. *Henkel R, Schreiber G, Sturmhoefel A, Hipler UC, Zermann DH, Menkveld R. Comparison of three staining methods for the morphological evaluation of human spermatozoa. Fertil Steril 2008;89:449-55.*
9. *Barroso G, Mercan R, Ozgur K, Morshedi M, Kolm P, Coetzee K, et al. Intra- and inter-laboratory variability in the assessment of sperm morphology by strict criteria: impact of semen preparation, staining techniques and manual versus computerized analysis. Hum Reprod 1999;14:2036-40.*
10. *Eustache F, Auger J. Inter-individual variability in the morphological assessment of human sperm: effect of the level of experience and the use of standard methods. Hum Reprod 2003;18:1018-22.*
11. *Guzick DS, Overstreet JW, Factor-Litvak P, Brazil CK, Nakajima ST, Coutifaris C, et al. Sperm morphology, motility and concentration in fertile and infertile men. N Engl J Med 2001;345:1388-93.*
12. *Kruger TF, Acosta AA, Simmons KF, Swanson RJ, Matta JF, Oehringer S. Predictive value of abnormal sperm morphology in in vitro fertilization. Fertil Steril 1988;49:112-7.*
13. *Brooks AK. How reliable are results from the semen analysis? Fertil Steril 2004;82:41-4.*