Improved Convential and Functional Seminal Parameters After Short Versus Long Ejaculation Abstinence

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BY
HIVA ALIPOUR

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By
Hiva Alipour

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The Faculty of Medicine
Department of Health Science and Technology,
Laboratory for Stem Cell Research
Fredrik Bajers Vej 7D, Aalborg Øst
Dk-9220, Denmark

Email: Hiva@hst.aau.dk

Dissertation submitted: 29-08-17

PhD supervisor: Prof. Ole Bjarne Christiansen
Department of Obstetrics and Gynecology
Aalborg University, Denmark

Assistant PhD supervisor: Prof. Gerhard Van der Horst
Department of Medical Bioscience
University of the Western Cape, South Africa

PhD committee: Associate Professor Josephus Dirk Nieland (chairman)
Aalborg University

Professor, Head of Division Jolanta Slowikowska-Hilczer
Medical University of Lodz

Professor,Senior Consultant MD, PhD Erik Ernst
Aarhus University

Moderator: Prof. Ole Bjarne Christiansen,
Aalborg University, Denmark

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CV

Hiva Alipour, graduated as a Doctor of Veterinary Medicine (D.V.M.) from Urmia Azad University (Urmia, Iran) in 2008. He then joined the embryology department of Royan Institute (Tehran, Iran) as a researcher and manager of the “Sperm Biology Lab.”. In 2013, he joined the “Biomedicine group” in the “Faculty of Medicine, Department of health science and technology” of Aalborg University (Aalborg, Denmark) as a PhD student and research assistant. He has since been actively involved in teaching and research in the area of reproductive health and medicine with focus on male fertility.

ENGLISH SUMMARY

The ejaculatory abstinence (EA) period can greatly affect sperm quality. However, the optimal EA period before delivering samples for semen analysis or infertility treatment is still a topic of debate. Previous studies on the influence of different abstinence times on sperm quality have provided controversial results. This can be due to these studies mostly focusing on study populations with sub-optimal sperm quality, while assessing semen parameters using different and often non-standardized methods, ultimately resulting in a lack of overall standardization and comparability.

This PhD project was designed to assess and compare the effects of short (two hours) versus long (4-7 days) ejaculatory abstinence periods on sperm quality parameters including sperm concentration, total sperm number in ejaculate, motility characteristics including kinematic parameters, morphology, and DNA fragmentation in normozoospermic men. Metabolomic profiles of the seminal plasma were also created to provide a possible insight into the mechanisms underlying the observed differences.

Normozoospermic male partners of couples undergoing *In-vitro* Fertilization (IVF) or Intra Cytoplasmic Sperm Injection (ICSI) at the Dronninglund fertility clinic (Aalborg University Hospital, Aalborg, Denmark) were asked to deliver two semen samples after a long (4-7 days) followed by a short (two hours) ejaculatory abstinence period. Sperm concentration, total sperm number per ejaculate, motility group categorization and kinematic details of the samples were performed using the SCA® (Sperm Class Analyzer) CASA system. The percentage of sperm with normal morphology was analyzed following “Spermblue” staining using the SCA. The DNA fragmentation index (DFI) (percentage of sperm with fragmented DNA) was assessed using the sperm chromatin structure assay (SCSA) and sperm chromatin dispersion (SCD) tests.

The results, demonstrated lower volume, sperm concentration and sperm number in total ejaculate; but, significantly increased percentages of progressive and rapid-velocity sperm, with a lower percentage of DNA fragmented sperm in samples obtained after two hours versus 4-7 days of abstinence. Metabolomic profiling of the samples using nuclear magnetic resonance spectroscopy demonstrated higher absolute amounts of pyruvate

and taurine per spermatozoa (metabolite concentration divided by sperm numbers) in ejaculates collected after the shorter abstinence time.

The higher absolute amounts of pyruvate as one of the most significant sources of energy required for mitochondrial function, and taurine as a capacitating and motility promoting factor, in the seminal plasma after shorter abstinence periods could be considered as an underlying mechanism behind the better motility observed in the second ejaculates. A shorter exposure to the oxidative stress caused by a possibly harmful seminal microenvironment during storage of the sperm in the cauda of the epididymis and vas deferens prior to ejaculation could also be considered as another underlying reason for the lower DNA fragmentation and generally better quality of the sperm after shorter abstinence periods.

Despite the lower volume, concentration and total sperm number in ejaculate after short abstinence, the higher percentages of spermatozoa with better motility and lower DNA damage increase, in theory, the possibility of selecting higher quality sperm for procedures not requiring many sperm (IVF, ICSI). Intrauterine inseminations requiring a higher number of motile spermatozoa may also benefit from the pooling of two consecutive ejaculates or using only the second ejaculate for treatment might improve fertilization rates. The lower levels of DNA fragmentation in the second sample could also result in reduced risk of miscarriage after IVF/ICSI.

However, further validation in large prospective randomized controlled trials, more purposely directed at males having problems to conceive when there appears to be no female factors, is needed to fully verify the potential advantage of using a second sperm sample in improving fertilization and pregnancy rates in assisted reproduction. Using several “omics” approaches comparatively and in combination, could allow for the identification of more metabolites and greater insight into the underlying mechanism behind the differences in sperm quality following different abstinence periods.

DANSK RESUME

Abstinensperioden efter ejakulation kan i høj grad påvirke sædkvaliteten. Ikke desto mindre diskuteres det stadigt, hvad der er den optimale abstinensperiode før indsamling af sædprøver til diagnostik eller fertilitetsbehandling. Tidligere studier af forskellige abstinensperioders betydning for sædkvaliteten har givet modsatrettede resultater. Dette kan skyldes, at de pågældende studier primært har fokuseret på populationer med suboptimal sædkvalitet samtidigt med at de har vurderet sædparametre ved hjælp af forskelligartede og ofte ikke-standardiserede metoder, som ultimativt resulterer i mangel på sammenlignelighed.

Dette Ph.D. studie var planlagt med henblik på, hos mænd med normal sædkvalitet, at bedømme effekten af korte (2 timer) versus lange (4-7 dage) ejakulationsabstinensperioder på mål for sædkvalitet så som spermiekoncentration og – antal, motilitetskarakteristika inklusive kinematiske parametre, morfologi og DNA fragmentation. Der blev også undersøgt profiler af sædplasmametabolomet.

Mandlige partnere med normal sædkvalitet fra par som gennemgik in-vitro-fertilisering (IVF) eller intracytoplasmatisk spermieinjektion (ICSI) på Dronninglund fertilitetsklinik, Aalborg University Hospital blev anmodet om at aflevere to sædprøver efter lang (4-7 dage) fulgt af en kort (2 timer) abstinensperiode.

Spermiekoncentration, totalt spermieantal, kategorisering iht. motilitet og kinematiske detaljer blev undersøg ved brug af SCA (Sperm Class Analyzer) CASA system. Procentdelen af spermier med normal morfologi blev analyseret ved SCA efter ”Spermblue” farvning. DNA fragmenterings index (DFI), som er procentdelen af spermier med fragmenteret DNA blev bedømt ved hjælp af sperm chromatin structure assay (SCSA) og sperm chromatin dispersion (SCD) test.

Der blev påvist et lavere volumen, spermiekoncentration og totalt antal spermier, men signifikant øget frekvens af progressivt og hurtigt bevægelige spermier og en nedsat frekvens af DNA fragmenterede spermier i prøver produceret efter 2 timers versus 4-7 dages abstinens. Metabolomisk profilering viste højere absolutte mængder af pyruvat og taurine pr.

spermatozoo (metabolitkoncentration divideret med spermieantal) i ejakulater indsamlet efter den kortere abstinenstid.

De højere absolutte mængder af pyruvat, som er en af de vigtigste energikilder for mitokondriefunktion, og taurin som en kapaciterende og motilitetsfremmende faktor i sædplasmaet efter korte abstinensperioder kan muligvis være en underliggende mekanisme for den bedre motilitet observeret i ejakulaterne efter kort abstinenstid. En mere kortvarig udsættelse for oxidativ stress forårsaget af et muligt fjendtligt miljø i epididymis og vas deferens før ejakulation kan være en baggrundsfaktor for den lavere DFI og generelt bedre sædkvalitet efter kortere abstinensperioder.

Trods det lavere volumen, spermiekoncentration og –antal efter kort abstinens, øger den højere frekvens af spermier med bedre motilitet og den lavere DFI i teorien mulighederne for at udvælge spermatozooer af høj kvalitet til procedurer, som kræver få spermatozooer så som IVF og ICSI. Ved intrauterin insemination, som kræve ret højere antal motile spermier, er det også muligt at det kan være en fordel at poole to konsekutive ejakulater eller kun anvende det andet ejakulat for at øge fertiliseringsraterne. Den lavere DFI efter kort abstinens kunne i teorien nedsætte risikoen for spontanabort efter IVF/ICSI.

Anvendelse af ejakulater efter kort abstinens ved iVF/ICSI elelr insemination skal dog valideres i store prospektive randomiserede kontrollede forsøg, som bør fokusere på infertile par, hvor der ikke ser ud til at være nogen risikofaktor hos kvinden. Anvendelse af adskillige ”omics” metoder individuelt og i kombination kan muligvis identificere flere betydningsfulde metabolitter og føre til større indsigt i mekanismerne, der ligger bag forskellene i sædkvalitet efter forskellige abstinensperioder

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Hiva Alipour
October 2017

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1. INTRODUCTION

1.1. THE SPERM CELL

The spermatozoon is the motile male reproductive cell, highly adapted to deliver the haploid male genome undamaged to the oocyte. Sperm are produced from the spermatogonia during the spermatogenesis process, which is estimated to be approximately 64 days in humans (Sadler and Langman, 2012). This process occurs in the seminiferous tubules and produces gametes with a haploid number of chromosomes (Hall and Guyton, 2011; Sadler and Langman, 2012).

The sperm cell can be morphologically/structurally subdivided into the head and flagellum (tail) sections, which are each responsible for a different physiological mechanism (Turner, 2003; Nieschlag *et al.*, 2010; Sadler and Langman, 2012).

The sperm head is composed of the acrosome and the nucleus. The acrosome holds the enzymes used to penetrate the zona pellucida, while the nucleus contains the male genetic material (Sadler and Langman, 2012). As the only type of cell designed to transfer from one individual to another, the packaging of the genetic material carried by the sperm has been specialized to protect the DNA from damage during transport and storage to the oocyte and to enable a complete rapid decondensation of the undamaged paternal genome in the ooplasm (Björndahl and Kvist, 2014). This specialized structure of the sperm DNA is based on the incorporation of protamines 1 and 2 that organizes the larger parts of the DNA in the nuclear matrix, along with some remaining “normal” histones in the minor DNA structures (Ward, 2010; Björndahl and Kvist, 2014). This alternate packaging structure allows sperm chromatin to be six times more condensed than the DNA in somatic cells (Ward and Coffey, 1991; Björndahl and Kvist, 2014). It has also been demonstrated that the damage caused to the DNA during the transfer of the sperm cannot be repaired before the DNA repair systems in the ooplasm gets access to the decondensed sperm DNA; Evidently, an increased number of DNA strand breaks in an individual sperm nucleus would result in an increased risk of errors in the repair process (Björndahl and Kvist, 2014).

The acrosome is located as a cap over the anterior part of the nucleus, in the most proximal part of the spermatozoa and makes up approximately 40-60% of the sperm head. The digestive enzymes stored in the acrosome, are

collectively responsible for the penetration and breakdown of the zona pellucida during fertilization (Wein *et al.*, 2015).

The connecting piece connects the sperm head and tail regions and contains the proximal centriole, which is a constituent of the spermatozoa centrosome involved in the creation of mitotic spindles during the first division from the zygote to the two cells stage (Sathananthan *et al.*, 1996; Chemes, 2012).

The complex yet highly stable cytoskeletal structure of the axoneme consists of 2 microtubules surrounded by 9 doublets, and extends throughout the flagellum. The outer 9 doublets have one Inner and one outer dynein arm, which create the motive force of the flagellum (Turner, 2003) and are surrounded by a sheath of mitochondria until one-fourth of the way down to the flagellum. These mitochondria provide the energy required by the dynein motor proteins accommodating the microscopic interactions within the axonemal complex located throughout the flagellum. This structure unit empowers a mechanical mechanism seen as a wave spreading backwards along the axis of the spermatozoa providing the required force for the motility of the sperm (De Jonge and Barratt, 2006; Wein *et al.*, 2015).

1.2. INFERTILTY AND ASSISTED REPRODUCTIVE TECHNOLOGY (ART)

Impaired fecundity and infertility have always been a health concern and are still considered as a significant clinical problem. Approximately 15–20% of couples worldwide, face the problem of infertility at some point in their life (Sharlip *et al.*, 2002), 40–50% of which are due to “male factor” infertility (Agarwal *et al.*, 2015) with approximately 2% of all men exhibiting sub-optimal sperm quality parameters (Kumar and Singh, 2015).

Currently, advanced ART techniques such as *in-vitro* fertilization and intracytoplasmic sperm injection (ICSI) allow for even severely impaired sperm to result in fertilization, clinical pregnancy and even live birth (Palermo *et al.*, 1993; Mansour *et al.*, 1995; Nagy *et al.*, 1995; Nijs *et al.*, 1996; Vandervorst *et al.*, 1997; Bungum, 2012). On the other hand, in spite of considerably improved live birth rates after ART in recent years, 19.9% of patients will still not succeed to have a child even after five cycles of ART (Wade *et al.*, 2015). Recurrent miscarriage, as one of the underlying reasons for such failures, has been recently suggested to be linked to sperm-related

factors, namely sperm DNA integrity (Larsen *et al.*, 2013). In that regard, the predictive value of the unique and functional characteristics of sperm for ART outcome, has been suggested to have great potential in assisting decision making process at clinic (Yetunde and Vasiliki, 2013; Oehninger *et al.*, 2014).

1.3. SPERM QUALITY ASSESSMENT

Evidently, a reliable and accurate assessment of sperm quality is a vital component of reproductive medicine and the first step in the diagnosis of the etiology of infertility in a couple; thereafter, selecting the best procedures to improve sperm quality (before and after ejaculation) and deciding on the overall treatment strategy in cases of male-factor infertility.

A set of predetermined values and parameters are often used to classify spermatozoa quality. In 1980, the World Health Organization (WHO) published the "WHO laboratory manual for the Examination and processing of human semen" (World Health Organization., 2010) as the first guideline for the standardization of procedures and reference values used in the analysis of human semen. Currently, the 5th edition of this guideline titled "World Health Organization laboratory manual for the examination and processing of human semen (WHO, 2010)" is used as a generally accepted guideline to standardize sperm analysis by the majority of the andrology and ART laboratories worldwide (WHO, 2010).

In addition to the conventional (manual) methods of sperm analysis, the 5th edition of the WHO guidelines also describes computer aided sperm analysis (CASA) as systems capable of assessing sperm concentration, motility, and detailed kinematics, as well as semi-automated morphology analysis. The WHO refers to the higher precision and quantitative data on the kinematic parameters of spermatozoa, as advantages of CASA over manual methods (WHO, 2010).

1.4. THE ASSESSMENT METHOD

1.4.1.Conventional sperm quality assessment (Subjective)

The results of sperm analysis (spermiogram) would normally include the sperm concentration, total sperm count, percentage of total motile sperm and sperm within different motility groups. Additionally, sperm analysis can also include the assessment of sperm morphology and DNA fragmentation index.

A continuous quality assurance (QA) program monitoring the quality and accuracy of the data on a regular basis, is considered as the best way to achieve reliable and acceptable sperm analysis results (WHO, 2010). Despite the availability of the recommended QA programs, several studies have demonstrated the presence of an inter-laboratory and inter-technician variation in the results of the conventional (subjective) sperm analysis (Auger *et al.*, 2000; Tomlinson, 2010; Rivera-Montes *et al.*, 2013; Daoud *et al.*, 2016).

1.4.2.Computer aided sperm analysis (Objective)

Until recently, difficulties in distinguishing spermatozoa from debris, by image processing software had made the assessment of sperm concentration and motility difficult and somewhat inaccurate (ESHRE, 1998) when performed by computer aided sperm analysis (CASA) systems. However, the newer generations of CASA systems have been shown to be capable of providing more precise and highly objective results compared with previous generations (Mortimer *et al.*, 2015).

These newer CASA systems can now provide standardization, speed, and precision, while reducing the potential for human error during performing of the tests or recording of the data at the same time. These systems also reduce the need for highly skilled professionals, while still providing repeatable, accurate and standardized results (Dearing *et al.*, 2014; Lammers *et al.*, 2014).

The “Sperm Class Analyzer (SCA®)” (Microptic S.L., Barcelona, Spain) CASA system has been assessed and validated as a tool for routine sperm analysis and promoted as providing more accurate and precise objective results with less analytical variance than manual methods for sperm concentration (Dearing *et al.*, 2014).

Some studies have suggested the detailed motility and kinematic characteristics of sperm obtained by CASA to serve as predictive biomarkers of fertilization rates *in-vitro* and *in-vivo* as well as time to conception (Liu *et al.*, 1991; Larsen *et al.*, 2000; Hirano *et al.*, 2001; Shibahara *et al.*, 2004).

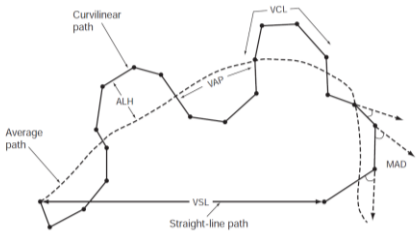
1.5. SEMEN QUALITY / CHARACTERISTICS

Many factors influence the quality of semen and spermatozoa, and consequently the ability for natural fertilization (Wein *et al.*, 2015). The most important factors that are used as potential biomarkers of the male fertility potential in this study are briefly explained in the subsequent subheadings:

1.5.1.Motility

Motility of a spermatozoa can be measured by their ability to swim through the seminal plasma and mucus in the female reproductive tract, to reach an oocyte (De Jonge and Barratt, 2006). Some of the motility characteristics assessed by CASA can provide a reliable estimation of the fertilizing ability of human sperm (Hirano *et al.*, 2001). Several studies have concluded that semen with a high proportion of spermatozoa with good motility demonstrates a high correlation with pregnancy rates. This makes spermatozoa motility an important parameter in semen analysis (Shibahara *et al.*, 2004; Freour *et al.*, 2009; de Araújo *et al.*, 2013).

CASA systems categorize the sperm into different types based on velocity and progression, calculated on the following kinematic details: average (WHO, 2010) path velocity (VAP, µm/s), straight line velocity (VSL, µm/s), curvilinear velocity (VCL, µm/s), Linearity (LIN), amplitude of lateral head displacement (ALH, µm), Straightness (STR), Wobble (WOB) and beat-cross frequency (BCF, Hz) defined at 50fps (WHO, 2010). Figure 1. demonstrates the standard terminology assessed by CASA.



$$STR = \frac{VSL}{VAP} \qquad LIN = \frac{VSL}{VCL} \qquad WOB = \frac{VAP}{VCL}$$

Figure 1. Standard terminology for variables measured by CASA.

1.5.2.Morphology

Sperm morphology is an important parameter in the assessment of sperm quality (Nieschlag *et al.*, 2010). Due to the high variation in spermatozoa shape and structure, the assessment of morphology is considered to be generally more complicated than other quality parameters (WHO, 2010). The definition of "normal morphology" used currently, is based on spermatozoa recovered from the postcoital endocervical mucus of the female reproductive tract (Fredricsson *et al.*, 1977; Menkveld *et al.*, 1990) and surface of the zona pellucida (Menkveld *et al.*, 1990; Liu and Baker, 1992).

Nevertheless, there is still no solid evidence about the possible influence of sperm morphology on success rates of natural or assisted reproduction, further emphasizing the need for more in-depth and large-scale studies (Kovac and Lipshultz, 2016).

1.5.3.Sperm DNA fragmentation

Parameters assessed by subjective or objective analysis including spermatozoon concentration, motility and morphology can provide a general assessment of sperm quality; but, no information about the integrity of the DNA as an important component of sperm quality evaluation.

The absence of a DNA repair mechanism in the spermatozoa means that the level of negative effect caused by a damaged chromatin, would depend on the level of damage and the capacity of the oocyte to repair it (González-Marín *et*

al., 2012). Studies demonstrating the negative effect of DNA breaks and molecular defects in the chromatin structure on fertility, led to the integrity of the sperm DNA gaining more attention as a predictor for male fertility (Evenson and Jost, 2000; Spanò *et al.*, 2000). Damage to the sperm chromatin integrity may be due to errors occurred at any step during spermatogenesis (Erenpreiss *et al.*, 2006) including unrepaired DNA breaks during the spermatogenetic remodeling and packaging of the chromatin, or exposure to genotoxic agents and oxidative stress (Sakkas and Alvarez, 2010). A higher incidence of DNA fragmentation and poor motility has been related to the risk of male-factor associated miscarriage (Khadem *et al.*, 2012), while single stranded DNA damage can be predictive of the sperm fertilization potential (Pregl Breznik *et al.*, 2013).

1.6. EJACULATORY ABSTINENCE PERIOD

Regardless of the standardization, QA programs and assessment methods, assessment of sperm concentration and motility classes are highly influenced by some confounding factors such as the frequency of ejaculations before the collection of sperm for quality assessment (Björndahl *et al.*, 2010). In order to achieve more standardized results reliable interpretation of the semen quality, the WHO guidelines have suggested an ejaculation abstinence period of 2–7 days prior to sampling (WHO, 2010). The suggested abstinence period (2-7 days) is based on results of some previous studies demonstrating that semen volume, sperm concentration and total sperm count are directly correlated with abstinence period up to 4-10 days (Macleod, 1951; Macleod and Gold, 1952; Gold and Macleod, 1956; Mortimer et al., 1982; Jørgensen et al., 2001, 2012), with the most distinct effect of the ejaculation abstinence observed in the first 24 hours (Hornstein et al., 1992; Matilsky et al., 1993; Tur-Kaspa et al., 1994; Makkar et al., 2001; Levitas et al., 2005; Francavilla et al., 2007).

Despite the established association between semen quality and ejaculation abstinence, it is still debatable whether the recommendation regarding abstinence is optimal for assisted reproductive technology procedures (Makkar et al., 2001; Levitas et al., 2005; Marshburn et al., 2010; Lehavi et al., 2014).

Several studies have reported a reduced success of fertility treatment if the samples used for treatment were obtained following short ejaculation abstinence periods (Frank et al., 1986; Levin et al., 1986; Tonguc et al., 2010).

On the other hand, this has been challenged by findings from studies of couples undergoing double intrauterine insemination (IUI) where an improvement in both sperm counts, motility and morphology were observed in ejaculates collected after a 24-hour abstinence period (Makkar *et al.*, 2001; Lehavi *et al.*, 2014). In line with this, higher pregnancy rates following IUI have been observed when using semen samples delivered with an abstinence period of less than two days, despite the lower total number of motile spermatozoa in these samples (Marshburn *et al.*, 2010).

Another study assessing sperm quality of a second sample provided only one hour after the first due to the poor quality of the first ejaculate for ART, also showed that the second sample was superior in quality to the first in 33% of the cases (Bar-Hava *et al.*, 2000). This study (Bar-Hava *et al.*, 2000) concluded that a second consecutive ejaculate can yield better results in a significant percentage of males with poor semen quality. A more recent study of ejaculates from men with oligozoospermia also detected a paradoxical improvement of sperm motility as well as sperm concentration when abstinence periods were as short as 40 minutes (Bahadur *et al.*, 2015) suggesting the acquiring of a second ejaculate as a possible alternate strategy in cases undergoing ART with poor seminal parameters (Juárez-Bengoa *et al.*, 2010; Bahadur *et al.*, 2015).

1.7. NUCLEAR MAGNETIC RESONANCE (NMR) SPECTROSCOPY

Assessment of the seminal plasma is not performed as part of the routine sperm analysis, unless there is a severe case which would necessitate the assessment of e.g. the concentration of zinc, citric acid, fructose, and alpha glucosidase. However, the composition of seminal plasma as the source of nutrition and protein required for the normal function of the spermatozoa may be considered as an important criteria in the assessment of sperm quality (Kovac *et al.*, 2013). The eclectic origin of seminal plasma, from the bulbourethral glands (1%), Sertoli cells (2-5%), prostate (25%–30%) and seminal vesicles (65%–75%), has confounded and slowed the discovery of biomarkers in this fluid (Kovac *et al.*, 2013).

Genetic or environmental cues can influence the metabolome and result in dynamic changes. The identification of this metabolome can provide an insight into the normal composition and physiology of cells while improving our understanding of the dysfunctions associated with pathological states (Jodar *et al.*, 2012; Wishart *et al.*, 2013; Castillo *et al.*, 2014a, 2014b).

Several chromatographic and spectrometric methods (Dunn *et al.*, 2005; Botros *et al.*, 2008) with different limitations and advantages have been used to assess metabolomic profiles. Nuclear Magnetic Resonance (NMR) spectroscopy can be used to identify novel compounds based on their unique spectral patterns. The non-selective, non-destructive, non-biased and quantitative nature of NMR makes it a great tool for metabolomics assessments (Emwas *et al.*, 2016; Ebrahimi *et al.*, 2017).

In spite of the prominent advances in metabolomics technology, the metabolomics of the human seminal plasma is understudied. In contrast to the over 6000 proteins identified in the human spermatozoa, (approximately 80% of the estimated spermatozoa proteome), only less than 100 metabolites have been found in the seminal plasma (Paiva *et al.*, 2015). There is a much higher number of proteins compared to metabolites in the human body (Schmidt, 2004; Kouskoumvekaki *et al.*, 2011; Wishart *et al.*, 2013), but the lower number of metabolites identified in seminal plasma and sperm can also be due to the ‘omics’ techniques not having been applied to their full potential in this field (Kovac *et al.*, 2013; Paiva *et al.*, 2015).

The slightest changes in the seminal plasma, even at the molecular level, can affect male fertility by altering the mechanisms controlling oxidative stress

(Wang *et al.*, 2009), chromatin condensation stabilization (Thacker *et al.*, 2011), DNA damage repair, promoting apoptosis (Zylbersztejn *et al.*, 2013) and exerting pro- and anti- inflammatory functions in addition to inflammatory response modulation (Juyena and Stelletta, 2012).Therefore, the metabolomic profiling of the seminal plasma has been considered as a fast and noninvasive diagnostic approach to evaluate male infertility (Hamamah *et al.*, 1998; Zhang *et al.*, 2015; Zhou *et al.*, 2016). However, there is no clear reference values for the seminal metabolites in normozoospermic cases and comparative analysis of seminal metabolomic profiles in male infertility have also received less attention to date (Zhang *et al.*, 2015).

1.8. AIMS AND HYPOTHESIS:

This PhD project was aimed to characterize the intra-individual differences in sperm quality in two ejaculates collected after 4-7 days (long abstinence) followed by two hours (short abstinence) of ejaculatory abstinence. The assessed quality parameters included morphology, concentration, motility and detailed kinematics, parameters using the SCA® CASA system. Additionally, sperm DNA integrity was assessed using the SCD and SCSA methods. Finally, nuclear magnetic resonance (NMR) spectroscopy was used to create metabolomic profiles of the seminal plasma in the collected samples. Comparative assessment of these profiles was used to gain a deeper insight into the underlying mechanisms behind the differences of sperm quality observed following different abstinence times.

1.8.1. Aims and hypothesis of the sub-studies

The project consisted of three components and the results are presented/published (Three papers) as described below:

- **Paper I**:

Assessed hypothesis: Sperm motility and kinematic parameters demonstrate better values in semen samples collected after long versus short abstinence.

- **Paper II**:

Assessed hypothesis: Difference in the metabolomic profiles of the seminal plasma can explain the differences in the sperm quality of samples collected after short versus long abstinence.

- **Paper III**:

Assessed hypothesis: Semen samples collected after short abstinence demonstrate better morphology and lower DNA fragmentation compared to long abstinence.

2. MATERIALS AND METHODS

2.1. STUDY DESIGN

The PhD project had a repeated-measures design, based on the assessment of semen samples from male partners of couples (43, 31 and 36 men for phase I, II and III of the study, respectively) attending for IVF/ICSI treatment at Dronninglund fertility clinic (Aalborg University Hospital, Aalborg, Denmark) between June 2014 and December 2016. The study was performed under approval by the scientific ethics committee of the North Jutland Region, Denmark (approval number N-20140023). An overview of the study design is shown in Figure 2, and described in detail below.

2.1.1. Sample collection

Semen samples used in this study were collected on the days when the patients were attending the hospital for the IVF or ICSI treatment. Since the first ejaculate was shared between this study and the fertility clinic's needs for treatment purposes, only patients with a sperm concentration above 15 million/ml in the first ejaculate were included in the study.

The participants were asked to maintain a minimum of 96 hours of ejaculatory abstinence before delivering the first sample. Semen collection was performed according to the procedure and precautions of the WHO 5 (2010) manual.

The samples used in this study were collected in a sterile collection cup by masturbation at home or in a private, quiet, adequately furnished room in the clinic. All samples were delivered or maintained at room temperature (<37°C) until reaching liquefaction.

The required volume for treatment and clinical purposes from the first ejaculate was separated and transferred to the clinical lab while the remaining volume was transferred to the research laboratory and used for this study. In the research laboratory, 1ml of the sample was separated and used for the collection of seminal plasma to be used in phase II, while the remainder was used for phases I and III, as it is further explained in the following sections.

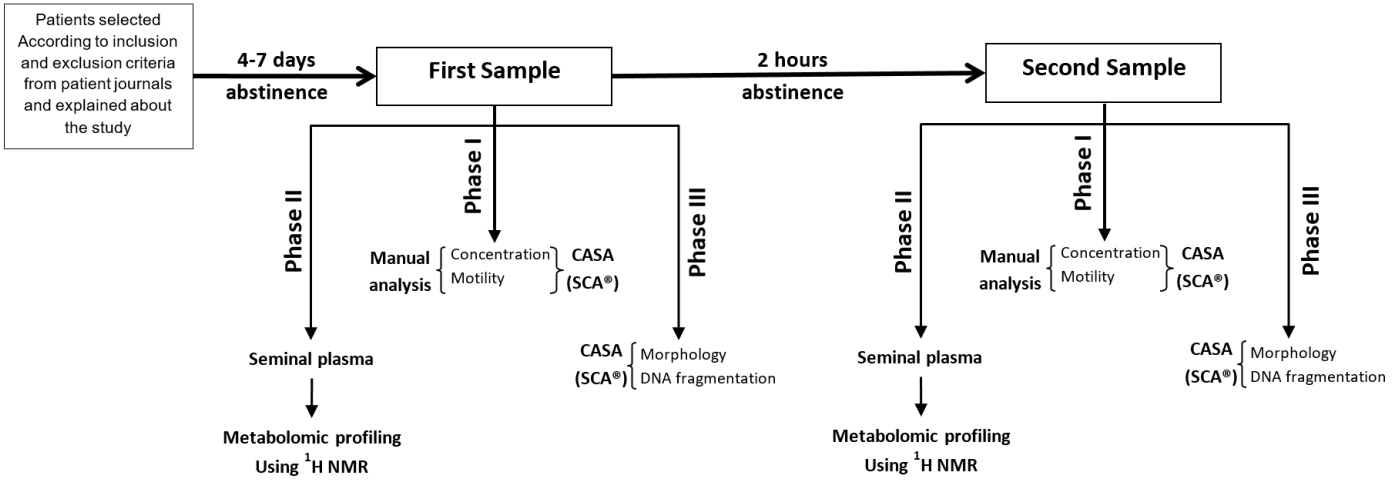


Figure 2. Schematic flowchart demonstrating the study design performed in three phases.

2.2. DESCRIPTION OF THE PARTICIPANTS

General participant information including name, birth date, name of partner, birth date of partner, height, weight, age, ethnicity, working conditions, education, used medications, previous health problems, fertility treatments etc. were obtained from the hospital reports or collected by the research nurse at the first consultation. The participants were selected based on the below general and cycle-specific inclusion and exclusion criteria:

2.2.1. Inclusion criteria:

1. Normozoospermic male partners of couples seeking infertility treatment, aged between 18 and 50 years
2. Able to understand and read Danish (to understand the basic project description and consent forms)

2.2.2. General exclusion criteria:

1. Ejaculatory disorders.
2. Impaired semen quality attributed to known genetic causes: abnormal karyotype, Y-chromosome microdeletions.
3. Impaired semen quality attributed to previous vasectomy, orchitis, unilateral orchiectomy, testicular cancer, other malignant diseases, metabolic diseases such as diabetes.
4. Psychological illness requiring chronic pharmaceutical treatment.
5. Cardiovascular disease.
6. The use of the following medications within the past 3 months prior to inclusion: antibiotics, antifungal agents, antidepressants, and other psychopharmacological treatments, cimetidine, cyclosporine, colchicine, allopurinol, or glucocorticoids.
7. Concurrent acute infection or inflammation.

Participants were at a later stage of the study excluded if there was an indication of use of inappropriate/illicit substance, an underlying significant medical condition or other conditions that were not recognized at the time of inclusion.

2.3. SAMPLE-SIZE CALCULATION AND STATISTICS

The main outcome measure used to calculate the sample-size for this study was the change in the "sperm DNA fragmentation (SDF) index" following sperm processing/selection, between the first and the second semen sample produced two hours after the first.

The required number of participants was calculated based on results of the study by Gosalvez et al. (Gosálvez *et al.*, 2011), in which the SDF was reduced from 17.0% (SD: 7.4) to 10.8% (SD: 6.3) in repeated semen samples produced with 3 hours intervals. In this study sperm donors with normal semen quality were investigated. Some of the men eligible for this study come from couples with unexplained infertility and therefore the mean SDF in the first ejaculate was expected to be higher than 17.0% and the standard deviation to be higher than 7.4 (Oleszczuk *et al.*, 2013). Assuming a mean SDF of 20% and a SD of 10 in the first samples and accepting a type I error of 0.05 and a type II error of 0.10, it can be calculated that a minimum of 30 double samples should be investigated to find a SDF reduction of 6% as found in the study by Gosalvez et al. (Gosálvez *et al.*, 2011). We aimed to include 43 men in the study in order to finalize with at least 30 men to ensure we could have all sperm parameters measured in both samples.

2.4. STUDY PHASE I

Phase I of the study was performed as described in appendix I (paper I). In brief, it is described here:

2.4.1.Semen sample analysis:

The semen volume was measured using a graduated pipette and visual assessment (color and viscosity) of the raw samples were recorded. The samples were allowed to liquefy at room temperature before they were divided into two parts. One part was used as raw semen and the second part was processed for sperm selection using routine gradient density centrifugation as explained below.

2.4.2.Sperm selection using density gradient

Semen samples from all ejaculates were processed using a discontinuous density gradient of silane-coated silica (Sydney IVF Sperm Gradient kit; Cook Medical, USA). Using a sterile 15 ml centrifuge tube (Falcon, USA), 1.5 ml of the '80%' gradient was gently layered below 1.5 ml of the '40%' gradient. The portion of the semen sample allocated for preparation by density gradient was gently layered on top of the density gradient. The tube was centrifuged at 300 g for 20 min to separate the spermatozoa. The supernatant was removed, leaving the lower 0.5 ml of the gradient containing the sperm pellet. Washing was performed twice with 1.5 ml Sydney IVF Fertilization Medium (Cook Medical, USA) at 300 g. After the second wash 500 µl of Sydney IVF Fertilization Medium was added to the pellet and used for analysis.

2.4.3.Concentration, Motility, and kinematic parameters:

Subjective analysis (Makler chamber)
All raw and density gradient processed samples were analyzed using the Makler chamber according to the laboratories conventional method as explained below:

A volume of 10 µl of well mixed semen was placed in a Makler counting chamber (Sefi Medical Instruments Ltd., Haifa, Israel) and evaluated using 20× (positive phase) magnification on a Nikon eclipse 50i (Nikon, Japan) microscope equipped with phase contrast optics. The number of spermatozoa counted in 10 random squares on the grid (of the cover glass) indicated the concentration in millions/ml. The percentage of motile/non-motile sperm was

also estimated based on the spermatozoa counted within the 10 random squares. Based on the assessed concentration and ejaculate volume, which were determined using a graduated plastic pipette following liquefaction, the total number of sperm in the ejaculate (total sperm count (TSC)) and total number of motile sperm (total motile sperm count (TMSC)) in ejaculate were calculated.

Objective analysis (SCA® CASA system)

At the same time, CASA was also used to quantitatively analyze the concentration and motility including the kinematic details based on the WHO criteria according to the below procedure:

A "Leja chamber slide" (10 µm deep) (Leja Products B.V., Nieuw Vennep, Netherlands) was filled with two micro liters of the liquefied sperm suspension and assessed at a total magnification of 100X using a Nikon E50i microscope equipped with a phase contrast condenser and a Basler sca780 (Basler, Germany) camera connected to a computer running the Sperm Class Analyzer (SCA®, Ver. 5.4, Barcelona, Spain) CASA software. The motility module of the SCA was used to analyze the concentration and detailed kinematic parameters of the spermatozoa. The detailed motion parameters provided by the SCA were used to categorize the sperm into different velocity and progression groups according to the WHO (WHO, 2010) reference values. The different motion and velocity parameters assessed by the SCA® have been illustrated in Table 1.

2.4.4.Statistical analysis

Basic description has been presented as means (± standard deviation), and medians (25th and 75th percentiles). Skewed data were normalized using natural logarithmic transformation before using paired samples *t* tests to compare results of consecutive ejaculates from individuals. The Wilcoxon matched pairs test was used to compare sperm concentration, semen volumes, TMSC, TSC and percentage of motile sperm. The association between semen volume and abstinence duration was evaluated using the Spearman's non-parametric correlation. Additionally, similarities in sperm concentrations analyzed using CASA and manual measurements were compared by Bland and Altman plots, subsequently validated by Passing and Bablok analyses. The MedCalc® statistical software (version 15.8, MedCalc Software, Ostend, Belgium) was used to perform the statistical analysis and $P<0.05$ was considered significant.

Table 1. The different motion and velocity parameters assessed by the Sperm Class Analyzer

Parameter	Unit	Description of the Parameter
Motility	%	Percentage of sperm in different motility groups based on velocity and progression
Concentration	$\times 10^6$/ml	Number of spermatozoa per milliliter
VCL	µm/s	Curvilinear velocity along actual swimming path
VSL	µm/s	Straight-line velocity along shortest path from start to end-point
VAP	µm/s	Average path velocity based on every 11^{th} frame of VCL path
LIN	%	Linearity of a curvilinear path, expressed as VSL/VCL
STR	%	Straightness, expressed as VSL/VAP
WOB	%	Wobble, expressed as VAP/VCL
ALH	Mm	Amplitude of lateral head displacement
BCF	Hz	Beat cross frequency based on VCL crossing VAP per second
Hyperactivated sperm	%	150< VCL (µm/s) <500; Lin (%)<50%; ALH (µm)>3,5*

** In most CASA systems, "ALH Max" is used which is approximately 2×ALH.*

(SCA®) CASA system. (Alipour *et al.*, 2017)

2.5. STUDY PHASE II

Phase II of the study was performed as described in appendix II (paper II). In brief, it is described here:

2.5.1.Nuclear magnetic resonance (NMR) spectroscopy

Approximately 1 ml of the semen sample was separated after liquefaction and centrifuged for 20 minutes at 3000 x g at 4°C, to separate the sperm from the seminal plasma. The top 500 µl of the supernatant was collected and frozen at -80°C until thawed for nuclear magnetic resonance (NMR) spectroscopy.

On the day of the NMR assessment, frozen samples were allowed to thaw slowly on ice before being centrifuged again for 30 minutes at 12100 x g to remove any possibly remaining spermatozoa. The supernatant was collected and mixed for 10 seconds using a vortex mixer. 100 µL of the collected seminal fluid was then added to 420 µL D2O and 60 µL 0.5 M phosphate buffer in a round bottom Eppendorf tube. The phosphate buffer is composed of 0.31 M Na2HPO4 and 0.19 M KH2PO4, pH 7, 0.23 M TSP-d4 (sodium-3-trimethylsilyl-[2,2,3,3-2H4]-propionate) and 20 mM NaN3. To minimize variations in NMR spectra, the samples were vortexed for 10 seconds and the pH was adjusted to 7.0±0.1 using a BRUKER BT pH titrator. D2O was added to reach a total sample volume of 600 µL; of which 550 µL was transferred to a 5 mm NMR sample tube. The samples were kept on ice throughout the whole process.

A Bruker AVIII-600 NMR spectrometer (Bruker Biospin, Germany and Switzerland) equipped with a cryogenic CPP-TCI probe at 600.13 MHz in connection with the Topspin 3.2pl5 program (Bruker Biospin, Germany) was used to acquire the 1H NMR spectra. The experiments were conducted at 310.1 K. CPMG (T2 relaxation-edited Carr–Purcell–Meiboom–Gill (Meiboom, Gill 1958)) (128 scans, 32768 complex data points, spectral width of 11.97 ppm and acquisition time 2.28 s. A relaxation delay of 2 s was used between each FID. A weak continuous wave irradiation ($\gamma B1/2\pi$ = 26.6 Hz) was used throughout the relaxation delay, at the water frequency (presaturation). The total spin-echo relaxation delay was 67.4 ms consisting of repeated (τ –π–τ) sandwiches, where τ was a delay of 0.4 ms and π was a 180°pulse of approximately 22 µs. Processing of spectra was carried out in Topspin 3.5.

1H-13C HSQC (Heteronuclear Single Quantum Coherence) and 1H-1H 2D-TOCSY (Total Correlation Spectroscopy) were recorded on selected samples for conformation of metabolites. These experiments were carried out with different numbers of FIDs, spectral widths, increments and mixing times to focus on different spectral regions. Spectra were corrected manually for the baseline and phase.

Identification and quantification of metabolites were performed using the Chenomx NMR suite 8.1 (Chenomx, Canada). In addition to the built-in Chenomx library, the Human Metabolome Database (Wishart *et al.*, 2007, 2009, 2013) was used to search for matching compounds and to verify resonance assignments.

2.5.2.Statistical analysis

The results of the NMR-based quantified metabolites (mg/dl) are presented as medians (25-75 percentiles). The Shapiro–Wilk normality test was used and data with non-normal distributions were normalized using natural logarithmic, or cubic-root transformation. The metabolite concentrations and sperm motility results of consecutive ejaculates from individual men were compared using the Paired samples t test.

The Spearman's non-parametric correlation was used to assess the association between semen volume, metabolite concentrations, and sperm concentration in the consecutive ejaculates, and the changes of the mentioned values in the consecutive ejaculates (Ej.2 – Ej.1). Statistical analyses were performed using MedCalc® software version 15.8 (MedCalc Software, Belgium).

2.6. STUDY PHASE III

2.6.1.Morphology

Air-dried smears of the semen were stained using “Spermblue” (Microptic S/L, Barcelona, Spain) based on the manufacturer’s instructions. The stained samples were assessed for normal/abnormal morphology using the morphology module of the SCA system at 1000X magnification.

2.6.2.DNA fragmentation (Halosperm kit)

Directly after liquefaction, 30 µl of each semen sample was separated and used for the assessment of DNA fragmentation using the sperm chromatin dispersion test performed using the Halosperm kit (Halotech, Madrid, Spain) while another 300 µl was frozen and maintained at -80 °C until being assessed for DNA fragmentation using the sperm chromatin structure assay (SCSA).

The sperm chromatin dispersion test (Halosperm)

The sperm chromatin dispersion test was performed using the Halosperm kit (Halotech, Madrid, Spain) according to the manufacturer’s instructions. In brief, unfixed sperm cells were bounded within two layers of an agarose micro gel on a slide. The slide was then incubated in an acid unwinding solution to “generate restricted single-stranded DNA (ssDNA) motifs from DNA breaks” (Fernández *et al.*, 2003). The membranes and proteins (protamines) (Fernández *et al.*, 1998, 2000) were then removed by immersing the slides in a lysing solution. The slides were then fixed in 70%, 90% and 99.99% ethanol and kept in a dark and dry place. When all samples were collected, the slides were stained using the brightfield staining kit (HT-BFS, Halotech, Madrid, Spain). After staining, the spermatozoa with big halos presented the sperm with low or without fragmented DNA, whereas a small or no halo demonstrated those with fragmented DNA. The halo sizes and fragmentation index were assessed using the “SCA® DNA fragmentation” module.

Sperm chromatin structure assay (SCSA)

A volume of 300 µl of the raw semen obtained from the first and second consecutive ejaculates was frozen at -80 °C and transported to the Reproductive Medicine Centre Skåne University Hospital (Malmö, Sweden) on dry ice. The frozen aliquots were allowed to thaw slowly on ice and assessed for sperm DNA integrity using the sperm chromatin structure assay (SCSA). A FACScan flow cytometer equipped with an air-cooled argon ion laser (Becton Dickinson, San Jose, CA, USA) was used to performed the

SCSA analysis as previously described by Bungum et al. (Bungum *et al.*, 2004).

Previous studies have demonstrated that freezing of sperm for later assessment of DNA fragmentation does not affect the SCSA parameters (Evenson *et al.*, 2002).

3. RESULTS

The mean (SD) and median (min-max) abstinence period before the collection of the first samples were 5.72 (1.16) and 6.00 days (4-7), respectively.

3.1. SEMEN VOLUME, CONCENTRATION, AND TOTAL SPERM NUMBERS

The descriptive statistics summary of Sperm concentration, total sperm number in ejaculate and total motile sperm number in ejaculate for the first and second samples both assessed using the Makler chamber and the SCA can be seen in Table 2.

Comparison of the sperm concentrations and percentages of motility groups reported by the SCA and manual assessment using the Makler chamber did not demonstrate any systematic error (refer to Appendix I, Supplementary section).

semen volume and abstinence period demonstrated a positive correlation (Spearman's rho=0.81, P<0.0001) resulting in a lower (P<0.0001) volume after two hours (Ejaculate 2) compared to "4-7 days" (Ejaculate 1) of abstinence period, with median (min-max) values of 2 days (1.2-4.5) vs. 3 days (2-6), respectively (Appendix I).

For 33 of the 43 men (77%), the percentages of progressively motile spermatozoa were higher in the raw samples collected after two hours' abstinence. 31 samples (72%) showed lower percentages of immotile spermatozoa (Table 3 and Figure 3).

The density gradient processed semen samples also demonstrated similar results with significantly higher percentage of progressively motile spermatozoa in 30 of 43 samples and lower percentages of immotile spermatozoa in 38 of 43 samples following two hours of abstinence.

The second ejaculate showed a lower average total sperm number in ejaculate in all the velocity, progression, and motility categories in the processed sample (Table 4).

Table 2. Sperm concentration, total sperm count and total motile sperm count in raw ejaculates after 4-7 days (ejaculate 1) and two hours (ejaculate 2) of abstinence assessed by the Makler's chamber and Sperm Class Analyzer (SCA). * demonstrates significant differences. (mill: millions; mill/ml: million per milliliter). Reproduced with permission from (Alipour et al., 2017).

	Makler *Median (25 – 75 percentiles)*	**SCA** *Median (25 – 75 percentiles)*	**P value**
Sperm concentration			
Ejaculate 1 (mill/ml)	40 (26 - 63)	41 (29 - 58)	0.40
Ejaculate 2 (mill/ml)	36 (20 - 62)	34 (18 - 50)	0.24
P value	0.09	0.003*	
Total sperm count			
Ejaculate 1 (mill)	124 (76 - 197)	120 (88 - 179)	0.62
Ejaculate 2 (mill)	70 (47 - 120)	61 (38 - 99)	0.081
P value	<0.0001*	<0.0001*	
Total motile sperm count			
Ejaculate 1 (mill)	75 (48 - 146)	88 (46 - 130)	0.08
Ejaculate 2 (mill)	50 (32 - 86)	44 (28 – 76)	
P value	0.0038*	<0.0001*	
Percentage of motile sperm			
Ejaculate 1 (%)	67 (51 - 77)	63 (49 - 79)	0.29
Ejaculate 2 (%)	75 (51 - 85)	74 (66 - 86)	0.075
P value	0.0136*	<0.0001*	

Table 3. Average percentage of sperm categorized by different criteria using the Sperm Class Analyzer (SCA) in raw and density gradient processed sperm from samples collected after 4-7 days of sexual abstinence and a second sample after 2 hours. The spermatozoa have been

SPERM AVERAGE PERCENTAGES

	Raw samples			Density gradient processed sample		
	4-7 Days abstinence	2 Hours abstinence	P value	4-7 Days abstinence	2 Hours abstinence	P value
W.H.O. classifications						
Type A	16 ± 11	22 ± 14	≤0.001	41 ± 19	45 ± 15	0.13
Type B	21 ± 9	25 ± 9	≤0.05	12 ± 5	18 ± 7	≤0.0001
Type A+B (PM)	37 ± 18	47 ± 18	≤0.001	54 ± 21	63 ± 1	≤0.01
Type C (NPM)	27 ± 7	26 ± 9	0.54	16 ± 6	16 ± 7	0.91
Type D (immotile)	37 ± 17	27 ± 14	≤0.001	30 ± 19	21 ± 12	≤0.01
Velocity						
Rapid	32 ± 18	42 ± 19	≤0.001	50 ± 21	58 ± 16	≤0.01
Medium	22 ± 7	23 ± 10	0.72	13 ± 5	14 ± 8	0.11
Slow	9 ± 2	8 ± 3	0.16	7 ± 3	7 ± 4	0.41
Static	37 ± 17	27 ± 14	≤0.001	30 ± 19	21 ± 12	≤0.01
Hyperactivated Spermatozoa	8 ± 7	11 ± 9	≤0.001	21 ± 13	23 ± 12	≤0.05

The presented data are "Mean ± SD";
Immotile: Non-motile sperm, NPM: Non-progressive motile sperm; PM: Progressive-motile sperm; Type A, B, C and D: Sperm motility according to the WHO 1999 criteria (WHO, 1999)

categorized based on progression, velocity, and W.H.O 4 criteria. Reproduced with permission from (Alipour *et al.*, 2017).

Table 4. Comparison of total sperm count (Mean ± SD) and median (max, min) of sperm categorized by different criteria using the Sperm Class Analyzer (SCA, Ver. 5.4) in raw and density gradient processed sperm from samples collected after 4-7 days of sexual abstinence and a second sample after two hours. The sperm have been categorized based on progression, velocity, and W.H.O 4 criteria. Reproduced with permission from (Alipour *et al.*, 2017).

	Total sperm counts (millions)					
	Raw samples			Density gradient processed sample		
	4-7 Days abstinence	2 Hours abstinence	P value	4-7 Days abstinence	2 Hours abstinence	P value
Progression (WHO 2010)	Median (25 - 75 percentiles)	Median (25 - 75 percentiles)		Median (25 - 75 percentiles)	Median (25 - 75 percentiles)	
PM	48.9 (17.7 - 87.8)	9.7 (4.7 - 23.8)	≤0.0001	6.2 (2.8 - 14.4)	2.3 (1.3 - 5.7)	≤0.0001
NPM	31.1 (19.1 - 49)	3.5 (2.1 - 6.9)	≤0.0001	3 (1.7 - 6.1)	0.6 (0.4 - 0.9)	≤0.0001
Immotile	35.8 (23.4 - 55.2)	4.7 (3.4 - 9.6)	≤0.0001	3.3 (1.8 - 6.1)	0.8 (0.4 - 1.3)	≤0.0001
Velocity						
Rapid	41.8 (12.8 - 83)	9.2 (4 - 22.4)	≤0.0001	5.8 (2 - 13.7)	2.2 (1.3 - 5.4)	≤0.0001
Medium	24.4 (15.6 - 38.3)	2.6 (1.5 - 5.5)	≤0.0001	2.6 (1.6 - 5)	0.6 (0.3 - 0.8)	≤0.0001
Slow	10.5 (6.1 - 14.6)	1.9 (0.9 - 3.1)	≤0.0001	1.3 (0.6 - 2.19)	0.2 (0.16 - 0.4)	≤0.0001
Static	35.8 (23.7 - 55.8)	4.7 (3.4 - 9.6)	≤0.0001	3.3 (1.8 - 6.3)	0.8 (0.4 - 1.3)	≤0.0001
W.H.O.						
Type A	14.4 (4.5 - 37.3)	7.3 (3.5 - 19.3)	≤0.001	3.3 (0.8 - 6.1)	1.6 (1 - 4.2)	0.1
Type B	21.1 (10.8 - 47.5)	2.6 (1.1 - 5.6)	≤0.0001	2.9 (1.7 - 7.3)	0.7 (0.4 - 1.4)	≤0.0001
Type C	31.1 (19.1 - 49)	3.5 (2.1 - 6.9)	≤0.0001	3 (1.7 - 6.1)	0.6 (0.4 - 0.9)	≤0.0001
Type D	35.8 (23.4 - 55.2)	4.7 (3.4- 9.6)	≤0.0001	3.3 (1.8 to 6.3)	0.8 (0.4 - 1.3)	≤0.0001
Hyperactivated spermatozoa	6.8 (1.9 - 20.4)	3.6 (1.8 - 10.4)	≤0.05	1.4 (0.4 - 3.2)	1 (0.4 - 2.6)	0.312

Immotile: Non-motile sperm, NPM: Non-progressive motile sperm; PM: Progressive-motile sperm;
Type A, B, C and D: semen profiles according to the WHO 1999 criteria (WHO, 1999)

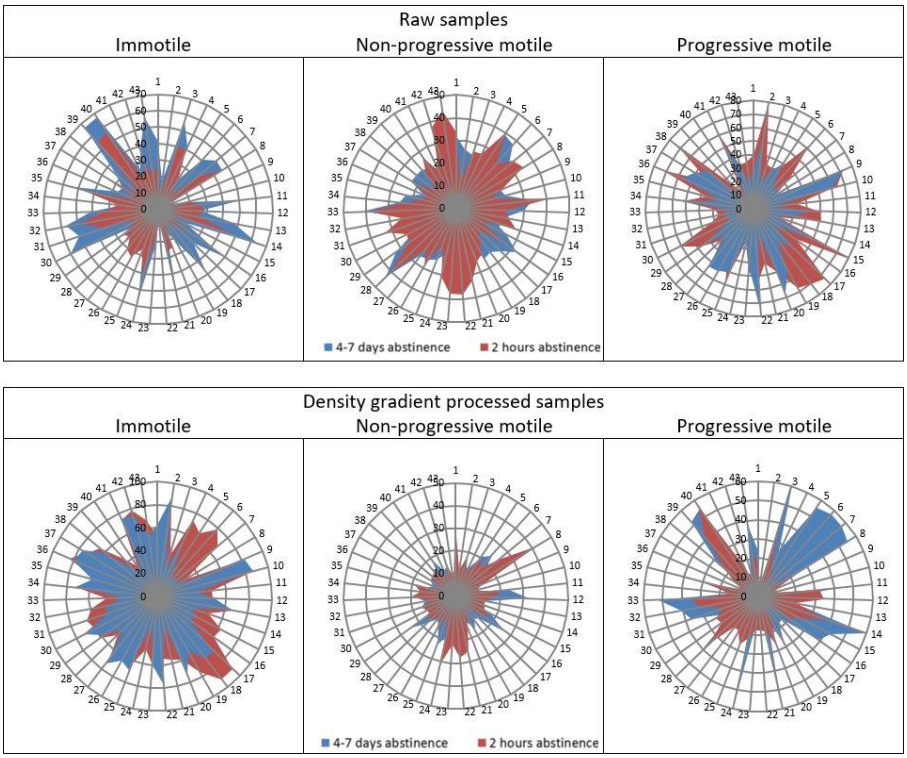


Figure 3. Sperm categorized by the Sperm Class Analyzer (SCA)based on progression (immotile, non-progressive motile, progressive motile) in raw (above) and density gradient processed (below) samples collected after 4-7 hours (in blue) and two hours (in red) of abstinence time. The graphs demonstrate the sample numbers on the perimeters and the radius demonstrates the percentage of sperm in the respective group. Immotile and progressive motile groups demonstrated significant differences. Produced with permission from (Alipour *et al.*, 2017).

3.2. MOTILITY

3.2.1.Motility according to WHO

Categorizing of the spermatozoa into four types (A, B, C and D) based on the WHO4 guidelines (WHO, 1999) demonstrated significantly lower total sperm count for all of the sperm types ($P \leq 0.001$ for type A and $P \leq 0.0001$ for types B, C and D) in the second raw ejaculate compared to the first (Paper I, Table IV). However, a significantly higher percentage of type A and B (according to WHO4) and progressively motile (type A+B, according to WHO5) sperm was seen in samples collected after two hours of abstinence while samples collected after 4-7 days of abstinence had a significantly higher number of type D sperm (Table 3).

Density gradient processed semen samples also demonstrated similar results with a significantly higher percentage of progressively motile spermatozoa and lower percentages of immotile spermatozoa after shorter abstinence in 30 and 38 of 43 samples, respectively (Table 3).

3.2.2.Motility based on velocity

Ejaculates collected after two hours of abstinence, demonstrated higher percentages of spermatozoa with rapid velocity and lower percentages of static sperm in samples collected after two hours of ejaculation abstinence (Table 3).

The density gradient processed semen samples also demonstrated a significantly higher percentage of rapid velocity ($P<0.01$) and significantly lower percentage of static ($P<0.05$) spermatozoa after short abstinence (Table 3). All the static, slow, medium, and rapid velocity sub-groups in both raw and density gradient processed samples had lower total counts in samples collected after short versus long abstinence.

3.3. KINEMATIC PARAMETERS

Raw samples demonstrated significantly (P≤0.001) higher median and quartiles of VCL and VAP) (Figure 5***Figure 5***) and median and quartiles of progressive swimming path (LIN, STR and WOB) values (Figure 6) in the second ejaculates (after two hours of abstinence). ALH and BCF did not show any significant difference between first and second ejaculates (p=0.052 and P=0.088 respectively) (Figure 6).

All of the average velocity parameters (VCL, VSL, VAP) (Figure 5) and derived kinematic parameters (LIN, STR, WOB, ALH and BCF) (Figure 6) in density gradient processed sperm demonstrated higher values after two hours of abstinence compared to samples collected after 4-7 days of abstinence when a 10% significance level was considered. However, this difference was only significant at the 5% level (P<0.05) in average VCL and average VAP.

The median and quartiles of the detailed kinematic parameters based on the slow, medium and rapid velocity categorization of the sperm in raw and density gradient processed samples, collected after 4-7 days of sexual abstinence and a second sample after two hours have been illustrated in (Table 5).

3.4. HYPERACTIVATION

Both the raw and the density gradient samples, showed more hyperactivated spermatozoa in samples obtained after two hours' abstinence (Table 3 & Figure 4). The mean (±SD) percentage of hyperactivated sperm compared to the initial ejaculate was 11% ± 9% and 8% ± 7%for the raw samples, respectively. The percentage of hyperactivated spermatozoa in the density gradient processed samples was higher than the raw samples regardless of abstinence time. The percentage hyperactivated spermatozoa was lower in the second ejaculate compared to the first in both raw ($P<0.001$) and after density gradient selection (Table 5).

The total count of hyperactivated spermatozoa was lower ($P = 0.029$) in raw samples of ejaculate 2; but, it showed no significant difference in the processed samples ($P<0.05$) (Table 5).

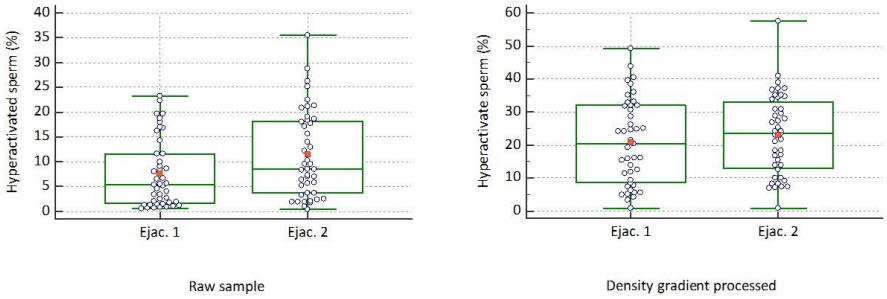


Figure 4. Box-and-Whisker plots demonstrating the percentages of hyperactivated sperm in samples collected after two hours of abstinence (Ejac. 2) compared to samples collected after 4-7 days of abstinence (Ejac. 1) in raw (left graph) and density gradient processed samples (right graph). Reproduced with permission from (Alipour *et al.*, 2017).

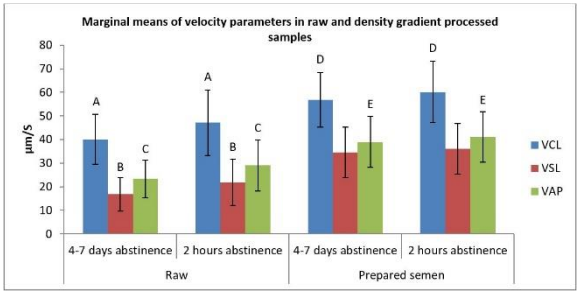


Figure 5. Three velocity kinematic parameters in raw samples collected after two hours and 4-7 days of abstinence. Parameters include curvilinear velocity (VCL), straight line velocity (VSL) and average path velocity (VAP). (Graph demonstrates mean ± SD; similar letters demonstrate significant pairwise differences). Reproduced with permission from (Alipour *et al.*, 2017).

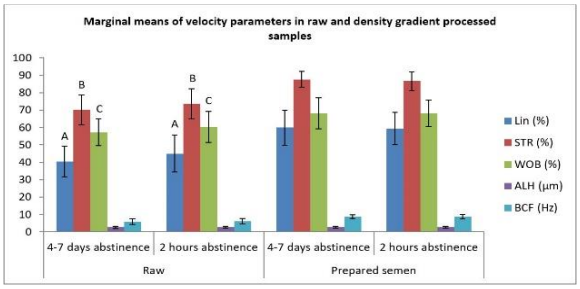


Figure 6. Kinematic parameters following density gradient selection in samples collected after two hours versus long abstinence. Parameters include Linearity (Lin), Straightness (STR), Wobble (WOB), Amplitude of Lateral Head displacement (ALH) and beat cross frequency (BCF). (Graph demonstrates mean ± SD; similar letters demonstrate significant pairwise differences. Reproduced with permission from (Alipour *et al.*, 2017).

Table 5. Detailed kinematic parameters assessed by the sperm class analyzer (SCA) for the slow, medium, and rapid velocity groups in raw and density gradient processed spermatozoa from samples collected after 4-7 days of sexual abstinence and a second sample after 2 hours. Data are presented as median (25th and75th percentiles) and compared using the paired t test. Reproduced with permission from (Alipour *et al.*, 2017).

	Raw samples			Density gradient processed sample		
	4-7 Days abstinence	2 Hours abstinence	P value	4-7 Days abstinence	2 Hours abstinence	P value
VCL (µm/sec)						
Slow	12.4 (12.3-12.5)	12.5 (12.3 - 12.6)	0.41	12.1 (11.0 - 12.4)	12.2 (10.5 - 12.5)	0.85
Medium	23.8 (22.9 - 24.1)	23.6 (23.1 - 24.1)	0.35	22.9 (21.6 - 24.0)	22.7 (21.5 - 23.8)	0.87
Rapid	61.1 (50.1 - 67.8)	64.5 (55.2 - 71.8)	≤0.0001	74.3 (66.5 - 80.6)	74.3 (66.2 - 81.9)	0.19
VSL (µm/sec)						
Slow	2 (1.7 - 2.6)	2.3 (1.9 - 2.6)	≤0.05	2.7 (2.1 - 3.6)	3.3 (2 to 5.2)	0.21
Medium	6.6 (5.3 - 7.7)	5.7 (4.8 - 6.9)	≤0.05	6.9 (5.3 - 8.6)	6.8 (5.4 - 8.8)	0.49
Rapid	27.4 (21.7 - 31.3)	32.2 (26.6 - 40.2)	≤0.0001	45 (41.0 - 52)	44.5 (38.5 - 50.0)	0.71
VAP (µm/sec)						
Slow	4.4 (3.9 - 5.5)	4.8 (4.1 - 5.6)	0.23	5.2 (3.9 - 6.2)	5.7 (3.8 - 7.4)	0.35
Medium	11.6 (10.1 - 13.7)	10.7 (9.1 - 12.6)	≤0.05	11.1 (9.8 - 12.6)	11 (9.3 - 13.5)	0.47
Rapid	35.9 (32.4 - 40.2)	41.5 (35.4 - 50.1)	≤0.05	49.2 (45.8 - 57.7)	49.3 (45.5 - 56.7)	0.57
STR (%)						
Slow	45.9 (42.9 - 52.8)	48 (43.5 - 53.6)	0.2	53.6 (48.5 - 69.7)	62 (49.9 - 76.0)	0.09
Medium	57.1 (51.3 - 59.7)	54.7 (51.6 - 57.3)	0.08	63 (54.5 - 70.9)	62.6 (56.8 - 68.5)	0.74
Rapid	74.9 (67.5 - 78.8)	79 (71.7 - 83.2)	≤0.001	89.8 (87.8 - 92.5)	89.7 (84.8 - 91.3)	≤0.01
LIN (%)						
Slow	15.9 (14 - 20.5)	18.7 (15.3 - 21.2)	≤0.05	23.3 (18.5 - 30.5)	27.8 (18.7 - 43.0)	0.18
Medium	28.2 (22.2 - 31.7)	24.7 (20.7 - 29.4)	≤0.01	31.2 (24.7 - 37.7)	29.5 (23.6 - 36.8)	0.52
Rapid	45.7 (40 - 50.0)	50.5 (42 - 57.2)	≤0.001	65.2 (53.7 - 72.1)	61.9 (53.8 - 69.1)	0.08
WOB (%)						
Slow	35.4 (31.4 - 45.1)	39.3 (34.0 - 45.1)	≤0.05	43.1 (34.8 - 51.4)	46.9 (36.5 - 59.6)	0.17
Medium	48.7 (42.6 - 57.1)	45.8 (40.4 - 52.8)	≤0.05	49.2 (43 - 55.8)	48.7 (40.0 - 56.4)	0.44
Rapid	62.4 (56.6 - 66.3)	63 (58.2 - 70.3)	≤0.01	73 (62.1 - 77.5)	71.3 (63.8 - 76.1)	0.41
ALH (Mm)						
Medium	1.7 (1.5 - 1.9)	1.7 (1.4 - 1.8)	0.15	1.4 (1.2 - 1.6)	1.3 (1.1 - 1.5)	0.28
Rapid	2.7 (2.3 - 3.5)	2.9 (2.5 - 3.4)	0.17	2.7 (2.4 - 3.2)	2.8 (2.5 - 3.3)	0.32
BCF (Hz)						
Medium	1.7 (1.3 - 2.5)	1.4 (1.1 - 2.1)	0.18	1.7 (0.9 - 2.4)	1.3 (0.9 - 2.3)	0.24
Rapid	7 (6.5 to 8.2)	7.5 (6.4 - 8.3)	0.36	9.6 (8.8 – 10)	9.3 (8.6 - 10.3)	0.53

Median (25th and75th percentiles); SCA ® default values were used to assess the motion parameters at 50fps. Curvilinear velocity (VCL, µm/s); straight line velocity (VSL, µm/s); average path velocity (VAP, µm/s); amplitude of lateral head displacement (ALH, µm); Linearity (LIN); Wobble (WOB); Straightness (STR); beat-cross frequency (BCF, Hz).

3.5. METABOLOMIC PROFILES

A typical sample of the acquired NMR spectra of seminal plasma demonstrating the within-subject comparison from ejaculates collected short and long abstinence periods can be seen in Paper II, Figure 1.

An overall number of 30 metabolites including 4 energy metabolites, 16 amino acids, and 10 other metabolites, were detected and quantified. The concentrations of the identified seminal metabolites, their absolute amounts (metabolite concentration X seminal volume), and absolute amounts of metabolites per 10^6 spermatozoa (absolute amounts / total sperm count) in samples collected after long and short ejaculatory abstinence can be seen in Paper II, Figure II.

Samples delivered after shorter abstinence demonstrated a significantly? higher concentration of pyruvate, but a significantly? lower concentration of fructose, N-acetylglucosamine, choline, acetate, O-acetylcarnitine, methanol, uridine and sn-glycero-3-phosphocoline. The remaining 21 metabolites showed no significantly different concentrations between the two ejaculates (Paper II, Table 2).

The absolute amounts of all metabolites demonstrated significantly lower values in the second ejaculate (Paper II, Table 2). However, the absolute amount per 10^6 spermatozoa of pyruvate and taurine was significantly higher following the shorter abstinence (Paper II, Table 2).

3.6. MORPHOLOGY

The sperm with normal morphology demonstrated median percentages (25-75 percentiles) of 12 (8-18) and 12 (6-18) for first and second ejaculates respectively; this difference was, however, not significant (P=0.72) (Paper III, Table 2).

3.7. DNA FRAGMENTATION

The statistical analysis of sperm DNA fragmentation in ejaculates collected after short and long abstinence periods assessed by the sperm chromatin dispersion (SCD) and sperm chromatin structure assay (SCSA), can be seen in (Paper III, Table 1).

The level of DNA fragmentation in ejaculates collected after a short abstinence period was lower than that collected after longer abstinence periods as assessed by both the SCD (P=0.03) and SCSA (P=0.0004) tests.

The SCSA also demonstrated a lower percentage of sperm with “moderate and high fragmentation” (P=0.0006 and P=0.011, respectively) following shorter abstinence.

Statistical assessments of method comparison using “Bland–Altman plots” and “Passing and Bablok linear regression” demonstrated a consistency of agreement between the SCSA and SCD tests. The results of these tests are presented in the supplementary section of Paper III.

4. DISCUSSION

This PhD project was designed to assess and compare the effects of short (two hours) versus long (4-7 days) abstinence periods on sperm quality parameters including sperm concentration, motility including kinematic parameters, morphology, and DNA fragmentation. Metabolomic profiles of the seminal plasma were also created to provide a possible insight into some of potential mechanism(s) of the observed differences.

4.1. SEMEN VOLUME, CONCENTRATION, AND COUNTS

The observed lower sperm concentration and semen volume consequently resulted in lower sperm number in total ejaculate after shorter abstinence (two hours) are in agreement with previous studies on men with sperm concentrations both above and below 15 mill/ml (Lampe and Master, 1956; Baker *et al.*, 1981; Poland *et al.*, 1985; Frank *et al.*, 1986; Levin *et al.*, 1986; Sauer *et al.*, 1988; Hornstein *et al.*, 1992; Matilsky *et al.*, 1993; Tur-Kaspa *et al.*, 1994; Makkar *et al.*, 2001; Levitas *et al.*, 2005; Sugiyam *et al.*, 2008).

A recent study detected a paradox increase in sperm concentration from 10 mill/ml in samples collected after 4 days of abstinence to 17 mill/ml in samples obtained after a 40 minutes abstinence period (Bahadur *et al.*, 2015). However, this increase was associated with a concomitant decrease of the mean semen volume from 2.7 to 1.1 ml. Although sperm number in total ejaculate were not reported, the mentioned figures indicated limited change in this parameter. Our study does not provide information to explain this finding. However, of the spermatozoa stored in the cauda of epididymis only about 50% are available for ejaculation (Björndahl *et al.*, 2010). Considering this, the reduced sperm concentration and counts observed in consequent ejaculates after a short interval may be associated with the poorly developed capacity of the sperm reservoir in humans (Sullivan and Mieusset, 2016). The possibly insufficient time for the transfer of spermatozoa from the more proximal epididymis sections to the cauda and vas deferens could be considered as another reason for this reduction in concentration and total count (Amann and Howards, 1980; Tommaso and William, 2013).

4.2. MOTILITY

The total number of motile spermatozoa in ejaculate obtained after only two hours of abstinence was significantly reduced in the raw ejaculates. This was obviously caused by the reduction in the total sperm number in ejaculate that were not compensated by the detected increase in the percentages of motile spermatozoa.

The influence of the length of ejaculatory abstinence period on sperm motility is still controversial. Different studies have reported diverse results including increased, decreased, or no change in percentage of sperm in different motility groups during different abstinence periods ranging from a few hours to 10 days (Sauer *et al.*, 1988; Check *et al.*, 1991; Magnus *et al.*, 1991; Blackwell and Zaneveld, 1992; Pellestor *et al.*, 1994). Some studies in men with a sperm concentration below 15 mill/ml have detected a higher frequency of motile spermatozoa in samples obtained after short abstinence periods (Levitas *et al.*, 2005; Bahadur *et al.*, 2015), while other studies did not find any difference (Sauer *et al.*, 1988). One study detected an increased percentage of motile spermatozoa with abstinence periods approaching 10 days (Magnus *et al.*, 1991). Studies in men with sperm concentrations above 15 mill/ml have reported significantly decreased the total number of motile sperm in ejaculate after 24 hours of abstinence compared with longer abstinence periods (2-4 days) (Matilsky *et al.*, 1993; Lehavi *et al.*, 2014).

To which degree the discrepancy in the published results reflect a true biological heterogeneity, intra-laboratory or inter-observer variations due to the well-known difficulties in assessment of the classical sperm motility parameters (Jørgensen *et al.*, 1997; Brazil *et al.*, 2004; Rivera-Montes *et al.*, 2013) cannot be answered by our results. However, we assume that our assessments based on the objective results obtained by the SCA are more precise and objective than conventional methods and previous generations of CASA (Dearing *et al.*, 2014; Mortimer *et al.*, 2015).

4.3. HYPERACTIVATION

Hyperactivation is a part of the complex process of sperm capacitation and is characterized by a motility pattern involving high velocity, high amplitude and marked lateral displacement of the head (curvilinear velocity greater than 150 µm/s; linearity less than 50%, and amplitude of lateral head displacement greater than 7 (Mortimer, 2000).

A former study has correlated higher fertilization rates to higher percentages of induced and spontaneous hyperactivated spermatozoa following conventional IVF (Pregl Breznik *et al.*, 2013). In this study, the ejaculates delivered after only two hours of abstinence, demonstrated a higher percentage of spontaneously hyperactivated spermatozoa. This may be related to the biochemical changes of the seminal plasma as a result of the limited time (two hours) available for the production and collection of secretions from the epididymis and prostatic glands for the second ejaculates (Elzanaty *et al.*, 2005). Metabolomic profiles of the seminal plasma from the consecutive ejaculates of this study demonstrated and outlined some of these changes (refer to Paper II).

The higher, but similar percentages of hyperactivated spermatozoa, in both ejaculates after density gradient selection, could be due to the induction of hyperactivation by the density gradient selection process itself.

The hyperactivation of the spermatozoa in this study was assessed by the SCA, while assessing other motility parameters were performed using a 10 µm deep Leja chamber. According to a study by Le Lannou et al. (1992), a chamber with a depth lower than 20 µm could constrain the development of the flagellar beat and alter the relative proportions of hyperactivated spermatozoa (Le Lannou *et al.*, 1992). However, percentages of hyperactivated sperm in the raw and density gradient processed samples of the first ejaculate in this study (using 10 µm deep chambers) were comparable to reference values and reports by previous studies, which had used 20 µm deep chambers (Burkman, 1984; Chan *et al.*, 1998; Kay and Robertson, 1998; Keppler *et al.*, 1999; Pregl Breznik *et al.*, 2013). This may be explained by findings of some recent studies (Suarez, 2016) that sperm maintain their optimal movements by adapting to their physical environment (Tung *et al.*, 2015a). The upstream swimming of sperm emerges via an orientation disorder-order transition. In addition, the hydrodynamic interactions of sperm as a front-back asymmetric swimmer with the wall (of the chamber in this study) is a sufficient criterion for upstream rotation (Tung *et al.*, 2015a).

A previous study demonstrated the ability of bovine sperm to quickly gain access and swim upstream efficiently in “microgrooves” (cross-sectional area of 20 × 20 µm) (Tung *et al.*, 2015b). Considering the longer and thicker flagellum (Smith *et al.*, 2009; Tung *et al.*, 2015b) and notably larger head of the bovine compared to human sperm (10×5×1 µm and 4.5×2.8×1.1 µm respectively), it might be reasonable to assume that human sperm can demonstrate hyperactivation in a 10 µm chamber by rotating the flagellar beat direction to stay in the horizontal pane within the chamber walls.

Assessment of sperm hyperactivation in 10 µm deep chambers may even result in better tracking of the detailed sperm movement using CASA by allowing a higher focus and visibility of the sperm during the movement; this assumption, however, requires further investigation.

4.4. KINEMATIC PARAMETERS

To date, “Paper I” is the first comparative report of the effect of short (two hours) and long (4-7 days) periods of abstinence on the motility and detailed kinematic parameters of normozoospermic samples (assessed by CASA). The data provided in this study can be used for comparison by future studies or as interim reference values for motility and kinematic details of normozoospermic samples after shorter abstinence periods.

Sperm motility and the regulation of different behaviors in human spermatozoa have in general been correlated with seminal fluid concentrations of different compounds like zinc, fructose, prostate-specific antigen and neutral alpha-glucosidase (Elzanaty *et al.*, 2005), small changes of pH, micromolar concentrations of Ca^{2+} (Peralta-Arias et al., 2015) and Ca^{2+} signals generated by CatSper and Ca^{2+} stores (Strünker *et al.*, 2011; Brenker *et al.*, 2012; Alasmari *et al.*, 2013). Such biochemical changes could be an explanation for the differences in sperm kinematics observed in our study.

The modifications required for the sperm to attain motility and fertilizing ability (Hunnicutt *et al.*, 1997; Jones, 1998a, 1998b; Belmonte *et al.*, 2000; Rejraji *et al.*, 2006; Tulsiani, 2006; Girouard *et al.*, 2011) including changes to flagellar beating (Sullivan and Mieusset, 2016) take place during the epididymal maturation of the sperm and are based on complex interactions between male gametes and epididymal secretions (Haidl *et al.*, 1994; Turner, 1995).

The length of the abstinence period could affect the transfer and storage time of the sperm in the epididymis and indirectly influence these complex interactions, consequently affecting the sperm kinematics.

The higher velocity and progression of the second ejaculate compared to the first might be related to the physiological and biochemical modifications of the spermatozoa during the transit through the epididymis as part of the maturation process, which do not affect the physical characteristics and density of the sperm.

The higher percentage of progressive motile and rapid-velocity spermatozoa seen in the second ejaculates in this study also supports previous studies suggesting that pooling consecutive samples would increase the numbers of available motile sperm (Tur-Kaspa *et al.*, 1990, 1994; Said and Reed, 2015). The finding could also indicate that even couples with no apparent male factor who are using ART may similarly benefit from providing a second ejaculate with an abstinence period of as short as two hours.

4.5. METABOLOMIC PROFILES

The metabolomic analysis of the seminal plasma in this study was based on untargeted Nuclear Magnetic Resonance Spectroscopy. 28 out of 30 identified metabolites, demonstrated no difference in the amounts per spermatozoa. However, when calculating the absolute amounts of seminal metabolites per spermatozoa (metabolite concentration divided by sperm count), pyruvate and taurine demonstrated significantly higher absolute amounts (mg) per 10^6 spermatozoa. The biochemical changes in the composition of seminal plasma, could be the reason for the better motility parameters of spermatozoa after shorter *versus* longer abstinence periods as suggested by previous studies (Bahadur *et al.*, 2015; Verze *et al.*, 2016). Pyruvate and lactate have been suggested as the most significant sources of energy for the function of sperm mitochondria and thereby key energy sources for motility and velocity parameters (Darr *et al.*, 2016). This increase in the absolute amounts of pyruvate and lactate may be considered as a possible explanation of the improved motility observed in the second ejaculates in the present study. Pyruvate has also been suggested to play a protective role against oxidative stress in somatic cells (Jagtap *et al.*, 2003; Hinoi *et al.*, 2006; Wang *et al.*, 2007), and spermatozoa in mice treated with cyclophosphamide (Bakhtiary *et al.*, 2015). The higher absolute amount of pyruvate per sperm in relation to the

mentioned protective effects could be considered as another potential explanation for the improved quality of sperm in the second ejaculates in this study. Seminal concentrations of fructose, as another energy metabolite, have shown no association with progressive motility (Elzanaty, 2007) or clinical usefulness in the assessment of defective sperm formation (Andrade-Rocha, 1999).

Taurine is one of the essential amino acids; but, it does not take part as a building block of proteins; it is, therefore, not categorized with the other amino acids (Wishart et al. 2013). Humans are capable of synthesizing taurine, but it is primarily originated from the diet (Holmes *et al.*, 1992). The seminal taurine has been shown to have an epididymal origin in animals (Holmes *et al.*, 1992); but, its origin in the human semen is still not clear. Taurine has been demonstrated to act as a sperm motility promoting factor (Boatman *et al.*, 1990; Yang *et al.*, 2010). It is also considered as a sperm capacitating agent (Meizel *et al.*, 1980; Meizel, 1985), and an antioxidative and membrane stabilizing factor (Alvarez and Storey, 1983; Mrsny and Meizel, 1985; Yang *et al.*, 2010), which can explain the possible beneficial effect of the higher absolute amounts of taurine per spermatozoa in the second ejaculate.

Proline and threonine have both been shown to negatively affect sperm motility in bulls (Roussel and Stallcup, 1967); but, these showed no difference in concentration, absolute amounts or absolute amounts per spermatozoa between the two ejaculates.

Uridine has been suggested to play a supportive role for the proper function of sperm during and after ejaculation (Niemeyer *et al.*, 2006). However, despite the enhancement of some velocity parameters in hyperactivated spermatozoa, addition of uridine was shown to reduce the percentage of motile spermatozoa (Niemeyer *et al.*, 2006), which is in agreement with the better sperm motility and lower concentration of uridine in the ejaculates delivered after shorter abstinence in this study.

The conversion of phosphorylcholine, catalyzed by prostatic acid phosphatase is considered as the source of the choline in the seminal plasma right after ejaculation (Mann, 1964). Even though the effect of choline concentrations on sperm motility parameters remain unclear; but, lack of the choline dehydrogenase enzyme, which is in charge of catalyzing the conversion of choline to betaine, leads to abnormal sperm mitochondrial morphology and reduced motility (Johnson *et al.*, 2010).

Total carnitine or carnitine acyltransferases have been demonstrated to have no effect on the respiratory capacity of sperm and thereby have no prognostic value (Brooks, 1979).

The lack of studies on the role of different seminal metabolites on sperm quality is clear and demands further studies focusing on the effect of different seminal concentrations of acetate, methanol, N-Acetylglucosamine and sn-Glycero-3-phosphocholine on sperm quality parameters.

Methionine, histidine, cysteine and aspartic acid (aspartate) have been identified and reported in previous metabolomic studies of the seminal plasma (Lynch *et al.*, 1994; Légaré *et al.*, 2013) and were, therefore, expected to be identified in this study. Hence, the missing of these metabolites could have been caused by technical issues and the complexity of identifying the many extensively overlapping resonances in a single pulse spectra from the untargeted ^{1}H-NMR (Lynch *et al.*, 1994) of the whole seminal fluid. The essential amino acids originating from the diet which were not identified in this study could be because they may not exist in the accessory sex glands, or not synthesized by the accessory sex glands in the case of the non-essential amino acids.

Considering the much longer period of spermatogenesis (Heller and Clermont, 1963) than the abstinence periods assessed in this study, the difference in the quality of the sperm in the two ejaculates cannot be associated with spermatogenesis, maturation and development processes. Sperm motility is initiated during the transfer from the epididymal conduit through the vas deferens at the time of ejaculation (Gupta *et al.*, 2014).

It may therefore be safe to consider the differences in the composition of the seminal plasma following long and short abstinence as an underlying cause for the different sperm motility parameters observed after different abstinence periods. Apart from a possible change in metabolic pathways, the difference in the composition of the seminal plasma between the two ejaculates could be due to the insufficient time available for the accessory glands to secrete and collect the metabolites before the second ejaculation. This hypothesis, however, requires further investigation.

4.6. MORPHOLOGY

Both of the ejaculates collected after short and long abstinence had a normal morphology above the WHO reference values (>4%) (WHO, 2010) with no significant difference after the different abstinence periods.

Several previous studies have challenged the misconception that extended sexual abstinence periods can improve sperm morphology (Pellestor *et al.*, 1994; Levitas *et al.*, 2005; Wongkularb and Sukprasert, 2013).

A former study has suggested that regular ejaculation may result in the production and ejaculation of immature and possibly sub-fertile sperm (Gosálvez *et al.*, 2011), while other studies (Carlsen *et al.*, 2004) and our results on the percentage of sperm with normal morphology suggest that the length of abstinence has no significant effect on sperm morphology. The reason for this could be that the spermatogenesis process in humans takes much longer (Heller and Clermont, 1963) than the abstinence time assessed in this study. Therefore, it would not be possible to differentiate the spermatozoa in the first and second ejaculate, based on the spermatogenesis or early stage maturation and development process, which may be an explanation for the similar normal morphology rates between the two ejaculates.

4.7. DNA FRAGMENTATION

Both the first and second ejaculates in this study demonstrated average percentages of DNA fragmentation below the threshold value (<30%) considered essential to achieve conception (Fernández *et al.*, 2003; Bungum *et al.*, 2004), and were also very similar to the threshold values for infertility of 18.90% for SCSA and 22.75% for the SCD suggested by a more recent study (Ribas-Maynou *et al.*, 2013). This was somewhat expected as the inclusion criteria for the study population focused on normozoospermic males of those couples attending for fertility treatment, thereby suggesting a higher chance of female factor in the participating couples. Nonetheless, the second ejaculates demonstrated a significantly lower mean percentage of sperm with DNA fragmentation regardless of the method used (SCSA or SCD).

In contrast to the subjective assessment of the results of the SCD test (presence and size of halo) in most other studies, the DNA fragmentation

module of the SCA CASA system was used to provide objective results for the SCD, which may explain the similarity of results between the SCSA and SCD tests seen in this study. A comprehensive study comparing several DNA fragmentation assessment methods demonstrated that both SCD and SCSA methods are suitable to discriminate between potentially infertile males (Ribas-Maynou *et al.*, 2013).

Sperm DNA damage can cause single-stranded and double-strand DNA breaks, as often seen in degraded sperm (García-Peiró *et al.*, n.d.; Cooke *et al.*, 2003; Ribas-Maynou *et al.*, 2012). Adverse reproductive outcomes have been linked to higher levels of sperm DNA fragmentation (Sakkas and Alvarez, 2010; Peluso *et al.*, 2013). The DNA fragmentation index (DFI) has even been suggested to hold a greater diagnostic value than the conventional semen analysis, and higher prognostic value for assisted reproductive techniques (Hull, 1992; Santiso *et al.*, 2007; Simon *et al.*, 2011, 2013; Cortés-Gutiérrez *et al.*, 2016). Higher levels of DNA fragmentation have also been correlated with increased complications in achieving pregnancy, recurrent miscarriage, and various childhood diseases (Cooke *et al.*, 2003; Aitken *et al.*, 2009; Brahem *et al.*, 2011; Zini, 2011; Absalan *et al.*, 2012).

Between ejaculations, the spermatozoa are stored in the cauda of the epididymis and vas deferens where the sperm function may be impaired in proportion to the storage time due to exposure to the oxidative stress possibly caused a harmful seminal microenvironment (Marshburn *et al.*, 2014; Agarwal *et al.*, 2016a). The higher seminal total antioxidant capacity (TAC) reported following shorter abstinence periods (1 vs 4 days) may be able to decrease the oxidative stress-induced sperm damage (Marshburn *et al.*, 2014) resulting in optimal levels of DNA fragmentation (Agarwal *et al.*, 2016b).

The lower DNA fragmentation index following the shorter ejaculatory abstinence observed in this study are in line with previous studies reporting lower sperm DNA fragmentation (Gosálvez *et al.*, 2011; Wongkularb and Sukprasert, 2013) and improved assisted reproduction outcomes when using spermatozoa collected after shorter abstinence periods (Jurema *et al.*, 2005; Marshburn *et al.*, 2010).

The higher percentage of hyperactivated sperm and lower levels of DNA fragmentation in the ejaculates collected after shorter (two hours vs 4-7 days) abstinence periods in this study (Paper I) (Alipour *et al.*, 2017) are in agreement with previous studies showing higher percentages of

hyperactivated spermatozoa with lower levels of DNA fragmentation (Torki-Boldaji *et al.*, 2017).

4.7.1.SCD vs SCSA

The availability of several different tests based on several different strategies has inevitably resulted in diverse reference values and interpretations of results (Agarwal and Allamaneni, 2005; Bungum *et al.*, 2006; Erenpreiss *et al.*, 2006; Ribas-Maynou *et al.*, 2013). A continuing debate on the accuracy and sensitivity of the different DNA fragmentation tests and their clinical value also exists (Agarwal and Said, 2003; Agarwal and Allamaneni, 2005).

In this study, the SCD test was performed immediately following liquefaction whereas an aliquot of the liquefied semen was cryopreserved at the same time and later used for the SCSA test. Previous studies have demonstrated that cryopreservation does not affect the results of the SCSA test (Evenson *et al.*, 2002).

The data from the SCD and SCSA tests have been previously shown to be correlated to different degrees (Evenson, 2016). Previous reports have validated and confirming the results of the SCD test (Velez de la Calle *et al.*, 2008). Nevertheless, the low number of spermatozoa (50–200 per sample) evaluated in the SCD assessment provides a lower statistical robustness than what is obtained in the SCSA which is based on the flow cytometric assessment of the DNA damage in 5000 spermatozoa (Evenson *et al.*, 2005).

4.8. STRENGHTS AND WEAKNESSESS

To our knowledge, this is the first project to comparatively report the intra-individual characteristics of sperm motility and kinematic details, morphology, DNA fragmentation in normozoospermic samples collected after 4-7 days versus two hours of abstinence. Additionally, metabolomics profiles of the seminal plasma of men stratified according to short or long abstinence periods provided an insight into the possible causes of the observed difference.

4.8.1.Phase I

The majority of previous studies had focused on the effect of different abstinence periods in samples with sub-optimal quality (concentration, morphology, DNA fragmentation, etc.). However, in this study the focus was placed on normozoospermic samples, which provided results allowing for more comprehensive interpretations and implications of a well-defined normozoospermic group. The results of this study also provided some objective interim reference values for the quality of normozoospermic sperm collected after short (2h) and long (4-7 days) abstinence periods. Furthermore, the objective assessments by the newer generation of CASA systems used in this study, allowed for more reliable, precise and objective assessments compared to the preceding generations (Mortimer *et al.*, 2015).

The ideal is that all semen samples are collected close to the laboratory. Some samples in this study were collected at home. However, care was taken to transfer, maintain, and analyze the collected samples from different sites in a consistent manner within one hour.

4.8.2.Phase II

The ongoing biochemical exchange between the spermatozoa and seminal plasma (after ejaculation and during liquefaction time), may also influence the concentration of the metabolites, further complicating the interpretation of NMR results (Apostoli *et al.*, 1997). Eliminating confounding factors such as the elemental exchange between the spermatozoa and seminal plasma during liquefaction time could provide a better understanding of the metabolomic profiles of consecutive ejaculates.

4.8.3.Phase III

In contrast to the subjective assessment of the results obtained from the SCD test (presence and size of halo) in most other studies, the DNA fragmentation module of the SCA CASA system was used to provide objective results for the SCD. The sperm DNA integrity of the two ejaculates was also assessed by both SCD and SCSA methods to provide a comparative and more reliable and precise results.

Possibly due to the selected study population, the level of sperm DNA fragmentations of the first ejaculates observed in this study were below the threshold value (<30%) suggested for successful conception (Fernández *et al.*, 2003; Bungum *et al.*, 2004); however, the effect of shorter abstinence periods on the integrity of the sperm DNA may be more evident in samples with initial sub-optimal DNA fragmentation levels.

5. CONCLUSION

The overall results based on normozoospermic samples, documented a lower volume, sperm concentration and sperm number in total ejaculate; but, significantly improved percentage of progressive and rapid-velocity sperm, and lower percentage of sperm with fragmented DNA in ejaculates obtained after an abstinence period of only two hours.

In spite of the lower volume, concentration and total sperm numbers in ejaculate, the higher percentages of spermatozoa with better motility and lower DNA damage in theory may increase the possibility of selecting higher quality sperm for procedures not requiring a large number of sperm (IVF, ICSI). Intrauterine inseminations requiring a higher number of motile spermatozoa may also in theory benefit from the pooling of two consecutive ejaculates or using only the second ejaculate for treatment that might consequently improve fertilization rates. The lower levels of DNA fragmentation in the second sample could also result in improvements of fertility success by reducing the risk of miscarriage.

Using a second sperm sample collected shortly after the first, has the potential advantage of improving the fertilization and pregnancy rates in assisted reproduction. However, further validation in large prospective randomized controlled trials, more purposely directed at males from couples having problems to conceive when there appears to be no female factors, is needed to fully verify this hypothesis. Using several “omics” approaches comparatively and in combination, could allow for the identification of more metabolites and greater insights into the underlying mechanisms of differences in sperm quality following different abstinence periods.

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APPENDIX: PAPER I - III

Paper I:

Alipour, H., Van Der Horst, G., Christiansen, O.B., Dardmeh, F., Jørgensen, N., Nielsen, H.I., Hnida, C., 2017. Improved sperm kinematics in semen samples collected after 2 h versus 4–7 days of ejaculation abstinence. Hum. Reprod. 32, 1364–1372. doi:10.1093/humrep/dex101

Paper II:

Alipour, H., Duus, R.K., Wimmer, R., Dardmeh, F., Du Plessis, S.S., Jørgensen, N., Christiansen, O.B., Hnida, C., Nielsen, H.I., Van Der Horst, G., Within-subject variation of Seminal plasma 1 metabolomics profiles following long (4-5 days) and short (2 hours) sexual abstinence periods, Submitted to "Hum. Reprod."

Paper III:

Alipour, H., Dardmeh, F., Van Der Horst, G., Bungum, M., Jørgensen, N., Hnida, C., Nielsen, H.I., Christiansen, O.B., Within-subject variation of sperm DNA fragmentation and normal-morphology following long (4-5 days) and short (2 hours) sexual abstinence periods, under submission to "Reprod Biomed Online"

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