University of Valencia

Department of Cellular Biology, Functional Biology and Physical Anthropology

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AN EXPERIMENTAL APPROACH TO OXIDATIVE STRESS EFFECT ON SPERM MOTILITY AND DNA FRAGMENTATION

DOCTORAL THESIS

By: SADEGHI KHOMAMI, Sara

Thesis supervisors: Dr SILVESTRE CAMPS, Miguel Angel,
Dr SOLER VAZQUEZ, Carles
Academic supervisor: Dr NUÑEZ DE MURGA, Javier

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ABSTRACT

In the study of male fertility in all species, the first and most basic parameters in sperm quality is its motility, count and morphology and how to maintain these important factors as a viable spermatozoon. Male infertility is usually associated with the presence of abnormal semen parameters. Spermatozoa with adequate rates of all these functional parameters could still be not able to fertilize and/or develop a zygote. Thus, it is clear that routine examination of the semen and the spermatozoa in the ejaculate cannot assess the process of capacitation or detect the DNA fragmentation or sperm mitochondrial dysfunction. Sperm DNA plays a critical role in normal embryo development as the genetic information passed on to the next generation depends on sperm DNA integrity. The research for this thesis was performed with the aim of using animal models to find the best way to measure DNA fragmentation in semen samples and evaluate the effect of oxidative stress on the viability and motility of spermatozoa. To this end, four experiments were performed, but only three of them are presented in this document. Firstly, in order to establish fish sperm activation conditions, it was necessary to study the effect of activation media in a range of osmolality and storage time and evaluate sperm motility parameters over time in zebrafish (*Danio rerio*). In Experiment 2, demonstrated how H_2O_2, as one the important oxidative stress agents, could affect the DNA fragmentation level and motility of zebrafish sperm. However, measurements of the halo area did not agree with the subjective sperm DNA fragmentation (SDF) rate. In this sense, it demonstrated, for the first time, an evolution of DNA fragmentation using morphometric criteria in human sperm (Experiment 3). The final experiment (Experiment 4) is included as an extended abstract in the Annexe 2. In this experiment, the Murciano-Granadina male goats were used to evaluate the correlation between semen cooled conservation conditions and DNA integrity, oxidation level, and mitochondrial activity in sperm using flow cytometry technologies.

The results of Experiment 1 demonstrated that the sperm motility rate was higher and the duration was prolonged with the activation medium with the highest osmolality. With regard to kinetic sperm parameters, we observed almost all the velocity parameters (VCL, VSL and VAP) increased significantly in a high osmolality medium. Total motility and velocity values of sperm were significantly lower after cool storage at 4°C for 24 h.

With regard to Experiment 2, we observed the motility of zebrafish sperm, mainly progressive motility, decreased as H_2O_2 increased. Moreover, SDF increased as the H_2O_2 concentration increased. However, measurements of the halo area did not agree with the subjective SDF rate. Using a SCD (sperm chromatin dispersion) test is a common way to determine the SDF level in cells. This test was based on using the presence of halo for SDF evaluation.

Finally, in Experiment 3, we demonstrated that the actual expansion of the halo is a continuous variable that cannot be reduced to one discrete value by choice. The DNA strands of sperm chromatin are highly condensed by protamines. In some mammals, chromatins contain a highly organised and compact structure which is more compact than the other species or human sperm.

RESUMEN

En el estudio de la fertilidad masculina en todas las especies animales, la movilidad espermática, junto a la concentración y morfometría son los parámetros principales y más básicos en la evaluación de la calidad de los espermatozoides. La infertilidad masculina habitualmente se asocia con tasas anormales de estos parámetros seminales. Aún teniendo, los espermatozoides tasas adecuadas de todos estos parámetros no se puede garantizar la capacidad de fertilizar y/o desarrollar un cigoto.

Por lo tanto, está claro que el examen rutinario del semen y los espermatozoides en la eyaculación no puede evaluar otros parámetros importantes en el proceso de fecundación como es capacitación espermática o detectar la fragmentación del ADN o la disfunción mitocondrial de los espermatozoides. El ADN del esperma juega un papel crítico en el desarrollo normal del embrión ya que la información genética transmitida a la próxima generación depende de la integridad del ADN del esperma.

Los trabajos de investigación de los que consta esta tesis se realizaron con el objetivo de utilizar modelos animales para esclarecer la mejor manera de medir la fragmentación del ADN de manera lo más objetiva posible, en muestras de semen y evaluar el efecto del estrés oxidativo sobre la viabilidad y motilidad de los espermatozoides. Para este fin, se realizaron cuatro experimentos, pero solo tres de ellos se presentan en este documento. En primer lugar, para establecer las condiciones de activación de los espermatozoides del pez, fue necesario estudiar el efecto de los medios de activación en un rango de osmolaridad y tiempo de almacenamiento y evaluar los parámetros de motilidad espermática a lo largo del tiempo en el pez cebra (*Danio rerio*).

En el Experimento 2, se estudió cómo el H_2O_2, como uno de los agentes importantes del estrés oxidativo, podría afectar al nivel de fragmentación del ADN y la movilidad de los espermatozoides en el pez cebra. Sin embargo, las mediciones del área del halo de la cabeza del espermatozoide no estaban coincidentes con la tasa subjetiva de fragmentación del ADN espermático (SDF).

En este sentido, se demostró, por primera vez, una evolución de la fragmentación del ADN utilizando criterios morfométricos en esperma humano (Experimento 3). El experimento final (Experimento 4) se incluye como un resumen extendido en el Anexo 2. En este

experimento, se usaron machos cabríos de Murciano-Granadina para evaluar la correlación entre las condiciones de conservación de semen refrigerado y la integridad del ADN, nivel de oxidación y actividad mitocondrial en espermatozoides utilizando la tecnología de citometría de flujo para su evaluación.

Los resultados del Experimento 1 mostraron como la tasa de movilidad de los espermatozoides era más elevada y que su duración se prolongaba cuando la activación se realizaba en el medio de activación con la osmolalidad más alta. Con respecto a los parámetros cinéticos, observamos que casi todos los parámetros de velocidad (VCL, VSL y VAP) aumentaron significativamente en un medio con la osmolalidad alta. La movilidad total y los valores de velocidad de los espermatozoides fueron significativamente menores después de su conservación a 4 ° C durante 24 h.

Respecto al Experimento 2, observamos que la movilidad de los espermatozoides de pez cebra, principalmente la movilidad progresiva, disminuyó a medida que aumentaba el H_2O_2. Por otra parte, la SDF aumentó a medida que aumentaba la concentración de H_2O_2. Sin embargo, las mediciones del área de halo no concordaron con la tasa subjetiva de SDF. El uso de una prueba de SCD (dispersión de cromatina de esperma) es una forma habitual para determinar el nivel de SDF en las células. Esta prueba se basó en el uso de la presencia de halo para la evaluación de SDF.

Finalmente, en el Experimento 3, demostramos que la expansión real del halo es una variable continua que no se puede reducir a un valor discreto por elección. Las cadenas de ADN de la cromatina espermática están altamente condensadas por las protaminas. En mamíferos, las cromatinas contienen una estructura altamente organizada y compactada aún más que otras especies o espermatozoides humanos.

RESUM

En l'estudi de la fertilitat masculina en totes les espècies animals, la mobilitat espermàtica, junt amb la concentració i morfometria són els paràmetres principals i més bàsics en l'avaluació de la qualitat dels espermatozoides. La infertilitat masculina habitualment s'associa amb taxes anormals d'aquests paràmetres seminals. Encara tenint, els espermatozoides taxes adequades de tots aquests paràmetres no es pot garantir la capacitat de fertilitzar i/o desenvolupar un zigot.

Per tant, és clar que l'examen rutinari del semen i els espermatozoides en l'ejaculació no pot avaluar altres paràmetres importants en el procés de fecundació com és la capacitació espermàtica o detectar la fragmentació de l'ADN o la disfunció mitocondrial dels espermatozoides. L'ADN de l'esperma juga un paper crític en el desenvolupament normal de l'embrió ja que la informació genètica transmesa a la propera generació depèn de la integritat de l'ADN de l'esperma.

Els treballs de recerca dels quals consta aquesta tesi es van realitzar amb l'objectiu d'utilitzar models animals per esclarir la millor manera de mesurar la fragmentació de l'ADN de la manera el més objectiva possible, en mostres de semen i avaluar l'efecte de l'estrès oxidatiu sobre la viabilitat i mobilitat dels espermatozoides. Per a aquesta finalitat, es van realitzar quatre experiments, però solament tres d'ells es presenten en aquest document. En primer lloc, per establir les condicions d'activació dels espermatozoides del peix, va ser necessari estudiar l'efecte dels mitjans d'activació en un rang d'osmolaritat i temps d'emmagatzematge i avaluar els paràmetres de mobilitat espermàtica al llarg del temps en el peix zebra (*Danio rerio*).

En l'Experiment 2, es va estudiar com l'H_2O_2, un dels agents importants de l'estrès oxidatiu, podria afectar el nivell de fragmentació de l'ADN i la mobilitat dels espermatozoides en el peix zebra. No obstant això, els mesuraments de l'àrea de l'halo del cap dels espermatozoides no estaven coincidents amb la taxa subjectiva de fragmentació de l'ADN espermàtic (SDF).

En aquest sentit, es va demostrar, per primera vegada, una evolució de la fragmentació de l'ADN utilitzant criteris morfomètrics en esperma humà (Experiment 3). L'experiment final (Experiment 4) s'inclou com un resum estès en l'Annex 2. En aquest experiment, es van usar

bocs de Murcià-Granadina per avaluar la correlació entre les condicions de conservació de semen refrigerat i la integritat de l'ADN, nivell d'oxidació i activitat mitocondrial en espermatozoides utilitzant la tecnologia de citometría de flux per a la seva avaluació.

Els resultats de l'Experiment 1 van mostrar com la taxa de mobilitat dels espermatozoides era més elevada i que la seva durada es perllongava quan l'activació es realitzava en el mitjà d'activació amb la osmolalitat més alta. Pel que fa als paràmetres cinètics, observem que gairebé tots els paràmetres de velocitat (VCL, VSL i VAP) van augmentar significativament en un medi amb la osmolalitat alta. La mobilitat total i els valors de velocitat dels espermatozoides van ser significativament menors després de la seva conservació a 4°C durant 24 h.

Respecte a l'Experiment 2, observem que la mobilitat dels espermatozoides de peix zebra, principalment la mobilidad progressiva, va disminuir a mesura que augmentava l'H_2O_2. D'altra banda, la SDF va augmentar a mesura que augmentava la concentració d'H_2O_2. No obstant açò, els mesuraments de l'àrea d'halo no van concordar amb la taxa subjectiva de SDF. L'ús d'una prova de SCD (dispersió de cromatina d'esperma) és una forma habitual per a determinar el nivell de SDF en les cèllules. Aquesta prova es va basar en l'ús de la presència d'halo per a l'avaluació de SDF.

Finalment, en l'Experiment 3, vam demostrar que l'expansió real de l'halo és una variable contínua que no es pot reduir a un valor discret per elecció. Les cadenes d'ADN de la cromatina espermàtica estan altament condensades per les protamines. En mamífers, les cromatines contenen una estructura altament organitzada i compactada encara mes que altres espècies o espermatozoides humans.

INTRODUCTION

1. Human infertility

Infertility remains both common and challenging among couples worldwide. As a clinical point of view couples can't achieve successful pregnancy after one year of regular sexual intercourse. An estimated 4–17% of couples seek medical treatment in order to resolve their infertility, and it is reasonable to presume that there are still lots of infertility cases that are not reported (Gnoth et al., 2005; Hamada et al., 2012a)

2. Male infertility factor

We have recently become aware of the importance of the male factor in the aetiology of human infertility and now recognise that defective sperm function is one of the most commonly defined causes of this condition. Male factor infertility is the important reason of infertility in 20–30% of infertile couples while contributing to 50% of cases overall. Approximately 7% of men worldwide are infertile (Bui et al., 2018; Mosher and Pratt, 1991)**.** The World Health Organization (WHO) periodically releases manuals for the laboratory examination and processing of human semen. The last update, which provided substantial improvements on how to assess the seminal parameters, was in 2010. In its latest edition, the semen analysis reference values are markedly lower than those of previous editions (Table1).

Table 1. Cut-off reference values for human semen characteristics as published in consecutive WHO manuals.

Semen characteristics	WHO 1980	WHO 1987	WHO 1992	WHO 1999	WHO 2010
Volume (mL)	ND	≥2	≥2	≥2	1.5
Sperm count (10^6/mL)	20–200	≥20	≥20	≥20	15
Total sperm count (10^6)	ND	≥40	≥40	≥40	39
Total motility (% motile)	≥60	≥50	≥50	≥50	40
Progressive motility	≥25	≥25%	≥25% (a)*	≥25% (a)	32% (a+b)
Vitality (% alive)	ND	≥50	≥75	≥75	58
Morphology (% normal forms)	80.5	≥50	≥30	14	4
Leukocyte count (106/mL)	<4.7	<1.0	<1.0	<1.0	<1.0

* Grade a=rapid progressive motility (>25 µm/s); grade b=slow/sluggish progressive motility (5–25 µm/s); ND = not defined

Inspired by Esteves et al., 2012

An initial male fertility examination includes a medical history, physical examination, general hormone tests and one or more semen analyses, which measure semen volume as well as sperm number, ability to move spontaneously, and quality of motion.

3. First steps in male infertility diagnosis and management and problems

The current methods of evaluation of male factor infertility remains routine semen analysis including: physical characteristics of semen, seminal volume and pH, sperm concentration, sperm motility, and morphology, leukocyte quantification, and fructose detection in cases where no spermatozoa are found and ejaculate volume is low. Currently, besides detailed medical history and thorough physical examination, routine semen analysis remains the sole of the evaluation of male factor infertility. Such practice is based on the fact that semen parameters such as sperm morphology, motility and concentration, have been shown to be significantly related to conception. In addition, the cost-effectiveness and non-invasiveness has led to extensive use of semen analysis in the initial evaluation of infertile men.

It is important to identify the limitations of semen analysis results in predicting the health and functional capacity of the male reproductive organs and cells. Many fundamental parts of the fertilisation process, such as capacity of sperm to transport to the oocyte, sperm capacitation with cervical mucus, and sperm interaction with the oocyte, cannot be assessed by conventional semen analysis. For this reason, it has been suggested that sperm function tests should be included in the semen analysis of individuals seeking fertility evaluation. Now it's the time to use more technological developments, in andrology field to bring effective and economical clinically useful sperm function tests to repair the shortcomings of routine semen analysis (Agarwal et al., 2008; Chong et al., 1983; Esteves et al., 2011; Hamada et al., 2012b; Lewis, 2007). However, this does not evaluate the full range of properties spermatozoa need to express to accomplish a successful fertilisation and pregnancy outcome.

Over the past decade, investigation of the role of sperm nuclear DNA integrity has become a growing research field. It has been suggested that DNA integrity may be a good predictor along routine sperm analysis. While high levels of sperm DNA damage are frequently associated with poor seminal parameters such as decreased in sperm count and motility and abnormal morphology (Irvine et al., 2000; Aitken and Baker, 2004; Schulte et al., 2010). Several studies have addressed the issue of the male infertility is strongly correlated with excess reactive oxygen species (ROS) in human semen (Aitken et al., 1989; Agarwal et al.,

2006). Furthermore, fertilised spermatozoa are capable of disrupting the epigenetic regulation of embryo development, but there are different opinions about which treatments are the best to avoid, damaged DNA sperm. Authors like Aitken and Baker, (2004) and Agarwal et al. (2006) believe that using ICSI (intracytoplasmic sperm injection) could result in reduced rates of embryo cleavage and pregnancy because all the functional processes associated with natural conception, such as capacitation, acrosome reaction, binding, fusion and penetration of zona pellucida, are bypassed. Conversely, authors such as Meseguer et al. (2011) and Lewis (2013) believe that sperm with DNA damage do not reduce success following ICSI because the oocyte can repair the DNA damage in women with no detectable problems even if the injected sperm is poor quality.

4. Semen analysis

The most common way to evaluate semen quality is based on subjective observations, which makes it almost impossible to compare sperm motility assessments performed by different laboratories; it also requires a great deal of time and experience. Semen evaluation has two main purposes, one related to male fertility in both human and other species, and the other for optimising insemination doses in livestock breeders. Even in complex infertility cases in which the female partner is also involved, the first and easiest evaluation step is performing semen analysis. With respect to livestock breeding and insemination centres, inaccurate estimates of sperm concentration could lead to faulty insemination doses (Bompart et al., 2018).

5. Computer-aided sperm analysis system

During the second part of the las century, several attempts were made to develop an objective method for evaluating sperm motility based on flagellar motility and sperm head position which were time consuming and impractical.

With the introduction of computer-aided sperm analysis (CASA) technology at the end of the 1970s, the aim was to overcome these problems and research approvals were obtained in both scientific and clinical fields. In an attempt to make assessment of semen quality more objective and detailed, tools for CASA have been developed. Overstreet et al. (1979) describe CASA as a simple, objective method for obtaining a well-defined and diversified set

of human sperm movement characteristics (CASA-mot). Using CASA-mot, several specific motility parameters describing the movements of spermatozoa in a more detailed manner can be obtained. Additionally, classification into motile and immotile spermatozoa can be based on well-defined velocity thresholds. Equivalent with efforts to improve the technical performance of CASA systems, it is important to investigate the biological relevance of CASA-mot parameters in terms of prediction of male fertility potential (Bompart et al., 2018; Larsen et al., 2000). The basic principle behind microscopy-based CASA-mot systems is a series of successive images of motile spermatozoa is acquired and analysed, which correlated with the flagellar movement patterns. CASA-mot systems provide a more objective sperm motility evaluation than visual estimation, as well as additional information such as kinetic variables. However, numerous factors unrelated to the semen samples could be influenced in sperm motility analysis, such as the commercial system we used and the effect of the technical setting, the number of frames, frame rate, and the type of chamber that was used for analysis. Sperm concentrations, diluents and volume and also sample factors which could introduce errors. Consequently, it is important to standardise the measurement conditions for sperm assessment to compare values among different studies (Del Gallego et al., 2017).

6. Sperm chromatin structure in mammal and fish sperm

Human and the most mammals sperm chromatin experiences extensive transforming during spermatogenesis, during which 85–95% of the histones are removed and replaced with protamines. Protamines are approximately half the size of histones. Sperm cell nuclei simply do not have the volume for this type of packaging. The replacement of most histones with protamines make a tighter packaging of the chromatin possible that is necessary for normal sperm function, and may help protect sperm DNA from damage during transport (Carrell, 2011; Ward and Coffey, 1991). In fish, zebrafish sperm genome packaged, in nucleosomes and histone variants and not protamines. The condensation of protamine-free zebrafish sperm is partially facilitated by increased linker histone levels (Wu et al., 2011).

6.1 . Sperm DNA damage and DNA fragmentation

During late spermatogenesis, DNA repair system downregulation could lead male germ cells to a higher possibility of DNA damage as the cellular mechanism that allows spermatozoa to undergo complete apoptosis is not functional during spermatogenesis. Additionally, abnormal spermatozoa originally assigned for elimination are able to escape apoptosis (Donnelly et al., 2000). However, the ejaculated sperm can contain both nuclear and mitochondrial DNA damage. The origins of this DNA damage could be explained if it is assumed that when spermatogenesis is interrupted by oxidative stress, it leads to the certainty of defective gametes with poorly remodelled chromatin that are particularly disposed to free radical attack. These defective cells tend to undergo an unusually shortened form of apoptosis to solve the problem associated with high amounts of superoxide generation by the sperm mitochondria. This leads to significant oxidative damage that eventually terminates in SDF (Aitken and Curry, 2011).

The major causes of DNA damage observed in spermatozoa are a result of oxidative stress (OS) and abnormal apoptosis (Aitken et al., 1989; Aitken and De Iuliis, 2010; Bui et al., 2018). Among various DNA anomalies, DNA fragmentation can be the most frequent sperm anomaly in infertile couples. There is now reliable evidence that a sperm containing fragmented DNA can be alive, motile, morphologically normal and able to fertilise an oocyte. Moreover, there is evidence that oocytes have the ability to repair damaged sperm DNA, but depending on the type of damage in sperm and also the quality of the oocyte itself, such as immaturity of oocyte, maternal age and other external factors, (González-Marín et al., 2012). Thus, it is important to understand the possible consequences of sperm DNA fragmentation (SDF) for embryo development, implantation, pregnancy outcome and the health of offspring, both naturally and by assisted reproductive technology (ART).

6.2 Different levels and types of sperm DNA damage

There are several different levels of sperm chromatin defects that are important to take into account:

a) Damage to the actual DNA integrity in the form of single-stranded or double-stranded DNA strand breaks.
b) Nuclear protein defects that may interfere with histone to protamine transformation and subsequent DNA compaction.
c) Chromatin structural abnormalities causing altered tertiary chromatin configuration.

6.3. Origin of DNA damage in spermatozoa

There are several mechanisms which could cause sperm DNA fragmentation and/or impaired chromatin integrity.

6.3.1 Intrinsic causes

a) Oxidative stress: the principal cause to defective sperm function is OS, which is a condition that reflects an imbalance between the systemic manifestation of ROS and a biological system ability to readily detoxify (antioxidant defences) the reactive intermediates or to repair the resulting damage (Agarwal et al., 2014). Spermatozoa are sensitive to OS because their plasma membrane contains a high percentage of polyunsaturated fatty acid. Spermatozoa are susceptible to a damaging process called lipid peroxidation because their plasma membrane containment. Peroxidation of polyunsaturated fatty acids generates lipid peroxides that have a detrimental effect on spermatozoa, disrupting DNA integrity. Lipid peroxidation occurs when electrons from plasma membrane lipids are exposed by ROS (Bui et al., 2018). The clinical significance of oxidative stress in the aetiology of defective sperm function was first indicated by Thaddeus Mann and colleagues, who recognised the ability of the antioxidant catalase to prevent motility loss in human sperm (Jones et al., 1979).

b) Apoptosis: as male germ cells experiencing changes into spermatozoa, lose their ability to undergo programmed cell death. Instead of engaging in a complete apoptotic response, spermatozoa is go through a different form of this process, leading to DNA fragmentation in the nucleus while remaining with the capacity to differentiate into mature functional spermatozoa that may still be capable of fertilising.

c) Chromatin remodelling or packing abnormalities: when histones are replaced by protamines during spermatogenesis, they undergo hyperacetylation, and then they replaced by protamines 1 and 2 (P1, P2). P1 and P2 are normally expressed in a 1:1 ratio in human sperm and provide a tight packaging of the sperm DNA. Abnormally high and low P1/P2 ratios are recognized to be associated with increased sperm DNA fragmentation, lower fertilisation rates, poor embryo quality and reduced pregnancy rates (González-Marín et al., 2012).

6.3.2 Extrinsic causes

Extrinsic causes of sperm DNA fragmentation and impaired chromatin integrity include: post-testicular OS, varicocele, bacterial infection, age, abstinence, temperature of testis, reactions to clinical procedures, medicine or vaccines, exposure to the chemical environment, and treatments and procedures of ART (González-Marín et al., 2012).

6.4. Reactive oxygen species

Reactive oxygen species, also known as free radicals, have at least one unpaired electron. They are oxidising agents generated as by-products of the metabolism of oxygen. Due to the unpaired electron in the outer shell, they form highly reactive molecules. ROS represents a collection of a broad range of radicals (e.g., hydroxyl ion [OH^-], superoxide ion [O_2^-], nitric oxide [NO], peroxyl [RO_2], lipid peroxyl [LOO], and Thiyl [RS^-]) and non-radical molecules (singlet oxygen [$^{-1}O_2$], hydrogen peroxide [H_2O_2], lipid peroxide [LOOH], and ozone [O_3]). When ROS are produced in large amounts, they have potentially toxic effects on sperm quality and function. Spermatozoa, like all cells living under aerobic conditions, constantly

face the Oxygen (O_2) paradox. O_2 is required to support life, but its metabolites, such as ROS, can make alteration in sperm function (Figure 1).

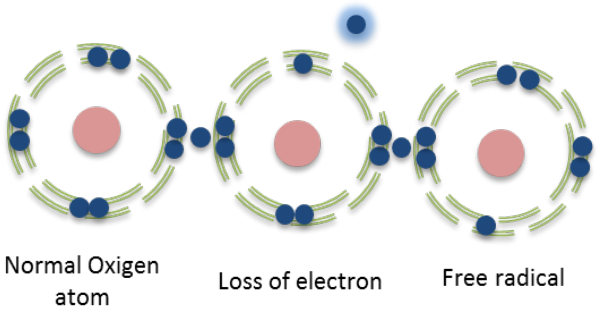

Figure 1: The creation of free radicals

6.5. ROS production by spermatozoa

Spermatozoa generate ROS in two ways:

- Presence of excess residual cytoplasm (cytoplasmic droplet): The defective cytoplasmic extrusion mechanism and immature and functionally defective spermatozoa could affect the enzyme which controls the intracellular availability of nicotinamide adenine dinucleotide phosphate (NADPH). NADPH is used as source of electrons to fuel the generation of ROS in spermatozoa.
- Mitochondrial activity: the mitochondrial system is the key source of ROS in spermatozoa from infertile men. The primary ROS generated in human spermatozoa is (O_2^-). This one-electron reduction product of oxygen secondarily reacts with itself in a dismutation reaction, to generate H_2O_2 (Agarwal, 2003).

6.6. ROS production by mitochondria

Sperm motility is the result of flagellar movement of the sperm tail, achieved by ATP-derived energy (adenosine triphosphate), produced in the mid-piece and located in mitochondria. Mitochondria are an important source of ROS within most mammalian cells. Mitochondrial respiration (respiratory chain) is the main biological source of superoxide anion radicals under physiological conditions. The respiratory chain generates a proton concentration gradient (change in pH) and a trans-membrane potential ($\Delta\Psi m$) across the inner membrane, which together allow the production of ATP by ATP synthase. In addition,

ROS can be produced in the mitochondrial electron transport chain by electron reduction of molecular oxygen. The superoxide (O_2^-) dismutation causes H_2O_2 generation within mitochondria. When respiratory activity is low, or when the NADH/NAD^+ ratio increases, it may lead to O_2^- or H_2O_2 (Murphy, 2009; Sanocka and Kurpisz, 2004). Davila et al. (2016) observed that ATP was not completely depleted by inhibiting mitochondrial function, and likewise, sperm motility was not completely abolished. They suggest that although disruption of mitochondrial function in stallion sperm leads to reduced motility, this could be a function of either reduced ATP production or increased reactive oxygen species production. Furthermore, they confirmed that mitochondrial ATP is essential for stallion sperm function. ROS does not appear to contribute to reduced sperm motility under uncoupling conditions, but ROS production becomes more intense when ATP synthase is inhibited. Mitochondrial inhibitors can both increase or decrease ROS production depending on the dose used.

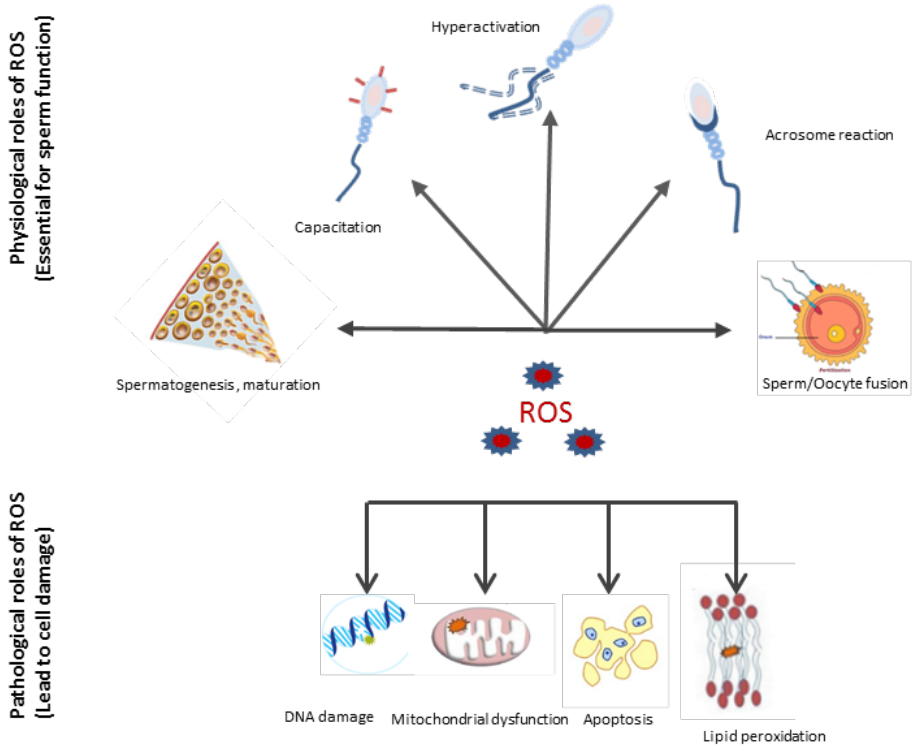

Figure 2: Reactive oxygen species (ROS) effect on physiological and pathological sperm function.

7. Effect of oxidative stress on male reproduction systems

7.1 Physiological roles of ROS in seminal plasma

Before, ROS were exclusively considered toxic to human spermatozoa. Aitken et al. (1989) was the first one proposed the idea that limited amounts of ROS can physiologically regulate some sperm. Low and controlled concentrations of physiological levels of ROS play an important role in the male reproductive system. Spermatozoa need a small amount of ROS for capacitation, hyperactivation, motility, acrosome reaction and fertilisation. In addition, an increase in cyclic adenosine monophosphate (cAMP) levels leads to an increase in sperm motility or hyperactivation (Figure 2).

It has been suggested that ROS take part in the regulation of nuclear maturation in spermatozoa (Agarwal et al., 2014; Sabeti et al., 2016). Spermatozoa exposed to excessive ROS levels become dysfunctional as a consequence of peroxidative damage to plasma membrane and are unable to initiate fertilisation. Any genetic damage induced by exposure of spermatozoa to oxidative stress is unlikely to be transmitted to the embryo. In ICSI patients, however, these barriers are removed and there is nothing to prevent spermatozoa with damaged DNA being injected directly into the oocyte (Irvine et al., 2000).

7.2 Pathological roles of ROS in seminal plasma

When the highly potent ROS overcomes the antioxidant natural defence systems and derange the balance between ROS and antioxidants, pathological defect occurs. Depending on the nature, cause and amount of the ROS attack, these defects may cause significant dysfunctions and damage to biomolecules such as lipids, proteins, nucleic acids, and sugars (Agarwal et al., 2014).

a) Lipid peroxidation: lipids are responsible for the fluidity of membrane layers and the changes that occur during capacitation in the female reproductive tract. Sperm plasma membrane is particularly vulnerable to lipid peroxidation by ROS due to the existence of a high concentration of polyunsaturated fatty acids. Lipid peroxidation can lead to loss of membrane fluidity and integrity and, as a result of this, reduces sperm-oocyte fusion. Furthermore, lipids can attack DNA by inducing base modifications, DNA strand breaks, DNA cross-links, and chromosomal rearrangements (Hosseinzadeh Colagar et al., 2013; Omid et al., 2010).

b) DNA damage: chromatin in the sperm nucleus is susceptible to oxidative damage, causing modifications and DNA fragmentation to the base. When DNA is minimally damaged, spermatozoa can undergo self-repair and potentially recover the ability to fertilise the oocyte and proceed with development. In cases where the oocyte repair machinery is not sufficient to repair DNA damage, the embryo may fail to develop or implant in the uterus and can be naturally aborted. DNA damage is a causative factor of apoptosis, poor fertilisation rate, high frequency of miscarriage, and morbidity in offspring (Agarwal et al., 2014; Chen et al., 2013).

c) Apoptosis: apoptosis plays a vital role in selectively destroying the pre-meiotic spermatogonia during the first round of spermatogenesis by preventing the over-production of germ cells from seminiferous tubules in response to ROS.

d) Mitochondrial DNA damage: there are approximately 70–80 mitochondria per spermatozoa in the midpiece of the flagellum. Mitochondrial DNA (mtDNA) is a small circular DNA located inside mitochondria and is encoding for 37 genes in humans. mtDNA have an important role in oxidative phosphorylation and ATP generation, which is important in motility of spermatozoa. ROS generation in mitochondria can attack sperm DNA and can affect the physiological functions of sperm (Amaral et al., 2013; Bui et al., 2018; Koppers et al., 2008). However, the generation of ROS from mitochondria is not straightforward; it is becoming an increasingly complex process. The involvement of ROS between different sites of ROS formation can vary significantly between different tissues and can also change depending on mitochondrial membrane potential. The mitochondrial electron transport chain creates an electrochemical gradient. This electrochemical gradient drives the synthesis of ATP and generates the mitochondrial membrane potential (MMP). Spermatozoa that exhibit high MMP generally have been shown to have high fertilisation capacity, as represented by increased ability for acrosome reaction, higher motility, and normal morphology (Andrabi, 2007). Spermatozoa with a larger level of damaged mitochondria cannot undergo a complete apoptosis and may result in a larger level of DNA fragmentation (Shamsi et al., 2008). Defects in sperm mitochondrial structure seem to correlate with decreased sperm motility in humans. However, it is important to note that, although mitochondria are present in the male

gamete, paternal mtDNA is generally not transmitted to the embryo in mammals after fertilisation (Amaral et al., 2013).

8. Factors involved in the creation of oxidative stress in ART

Besides of the internal and natural causes of DNA fragmentation, extrinsic causes that could take place in human and in animal reproduction sciences also exist. Most important factors affecting are:

a) Sperm collection methods, diluents, extenders and post ejaculation treatments: using different types of diluents in semen collection and culture media, centrifugation, breeding season or non-breeding season, the time gap between semen collection and semen analysing procedures. Also, differences are found between species and among individuals of the same species.
b) Storage temperature and cryopreservation: gamete cryopreservation is widely used for a variety of purposes, such as fertility preservation prior to chemotherapy treatment, donor or conjugal sperm cryopreservation, or research. Conversely, semen cryopreservation is an effective technology for improving livestock breeding programs. As noted previously, it is important to minimise all the negative effects of cryopreservation to preserve the better quality of the thawed sample. Cryopreservation not only reduces sperm motility and vitality, it affects DNA damage in sperm cells caused by the ROS (Ribas-Maynou et al., 2014; Spanò et al., 1999; Zribi et al., 2010).

There is no general cure or treatment for OS-induced male infertility, but prevention actions such as having a cost-efficient and comprehensive laboratory test can be carried out for early intervention. The routine laboratory techniques used to measure OS are a result of direct measurement of ROS free radicals or reactive nitrogen species, or indirect measurements through accumulative results of oxidised products, such as DNA fragmentation level in sperm cells or through mitochondrial activity from NADPH reduction in mitochondrial DNA (mtDNA).

9. DNA fragmentation measurement techniques

Several techniques exist to detect sperm DNA fragmentation. Over the years there have been increasing numbers of tests to evaluate and measure levels of DNA integrity. The use of these tests has been driven largely by the emergence of ARTs in humans and domestic/non-domestic animals. Both direct and indirect assay methods have been used to measure sperm DNA damage. More common direct methods for detecting DNA breaks include the TUNEL and comet assay while the most common indirect methods for evaluating DNA damage.

a) Sperm chromatin structure assay (SCSA): a sperm chromatin structure assay is a flow cytometric test where sperm DNA breaks can be evaluated indirectly thorough the DNA denaturability. This assay is based on the inference that a damaged chromatin structure is more prone to acid or heat denaturation. The parameters obtained from this method are called the DNA fragmentation index and are a measure of DNA denaturation. This is a powerful technique in extensive clinical research, especially in human samples, but it requires expensive instrumentation for optimal analysis (Andrabi, 2007).

b) TUNEL: the terminal deoxynucleotidyl transferase-mediated (TdT) deoxyuridine triphosphate (dUTP) nick end labelling assay (TUNEL) is a direct quantification of sperm DNA breaks. dUTP is incorporated at single-stranded and double-stranded DNA breaks in a reaction catalysed by the enzyme TdT. The DNA breaks based on the incorporated dUTP are then labelled and can be measured using bright field or fluorescent microscopy or flow cytometry. Then, sperm is classified as TUNEL positive or negative and expressed as a percentage of the total sperm in the population (Bungum et al., 2011; Fernández et al., 2005; Schulte et al., 2010).

c) Comet assay test*:* another test for direct assessment of sperm DNA breaks is the comet assay technique. This is a straightforward method for assessing DNA strand breaks in eukaryotic cells and the methodology is relatively simple. In a single-cell gel electrophoresis, the amount of low molecular weight DNA is assessed by

measuring the length and area of the comet formed during electrophoresis of spermatozoa. Sperm DNA breaks move away from the head region to form comets following electrophoresis, while at intact DNA remains in the actual head position. Decondensed sperm are suspended in an agarose gel, subjected to an electrophoretic gradient, stained with fluorescent DNA-binding dye, and then imaged with imaging software. Low molecular weight DNA, short fragments of both single-stranded and double-stranded DNA, will migrate during electrophoresis, giving the characteristic comet tail. High-molecular weight intact segments of DNA will not migrate and remain in the head of the "comet" (Andrabi, 2007; Cortés-Gutiérrez et al., 2014; Schulte et al., 2010).

d) Sperm chromatin dispersion test: Fernández *et al.* (2005) developed an easy and fast method for determination of DNA fragmentation in sperm cells in human and other species, called the sperm chromatin dispersion (SCD) test. Intact spermatozoa are immersed in an agarose matrix on a slide, treated with an acid solution to denature DNA that contains breaks, and then treated with lysis buffer to remove membranes and proteins. The agarose matrix allows work with unfixed sperm on a slide in a suspension-like environment. Removal of nuclear proteins results in nucleoids with a central core and a peripheral halo of dispersed DNA loops. Those sperm nuclei with elevated DNA fragmentation produce very small or no halos of DNA dispersion, whereas those sperm with low levels of DNA fragmentation release their DNA loops and form large halos (Fernández et al., 2005). The SCD test is a "simple" method in kit form. Unlike all the other tests, it measures the absence of damage rather than the damaged DNA in sperm. Nevertheless, the inter-observer reliability of subjectively categorising the halos is a limitation of the SCD technique. The evaluation of DNA damage in this technique as with other seminal parameters is a subjective evaluation, but the actual expanse of the halo is a continuous variable that cannot be reduce just by a choice. Using a classification matrix of different subpopulations could be used for the future definition of this current subjective evaluation.

10. Assisted reproductive technologies in human and animal systems

There have been 3,799 studies reported worldwide over the past five years concerning male infertility. While 2,740 of these aimed to reveal male infertility in humans, the rest were animal-based studies. Animal Models and Human Reproduction (Schatten and Constantinescu, 2017) is a comprehensive reference that reflects the latest scientific research being undertaken in human reproductive biology utilising domestic animal models.

a) Zebrafish, a model for fertility research: there is a significant growth in the use of zebrafish as models for genetic and fertility fields of study. *Danio rerio*, commonly known as zebrafish, is a tropical freshwater fish. It was previously a well-known aquarium fish, and has transformed into an indispensable animal model for scientists. The numerous advantages and characteristics of this small animal have never failed researchers when using this model for scientific projects. Perhaps zebrafish's low cost and ease of maintenance make it popular in the laboratory. Nonetheless, the fact that the well-characterised gene functions of zebrafish display a high degree of similarity with human genes have certainly improved the confidence level and potential implications of research findings. Zebrafish play a fundamental role as a model in evolutionary science, genetics, neurobiology development, biology, drug discoveries and even environmental monitoring efforts. In fertility and reproductive science, zebrafish have grown as a promising model because of their developmental and physiological advantages. The short cycle of the reproductive period and the transparency of these animals in the early stages, easy and repetitive sperm and zygote collection without any scarification, allow for more economic and comprehensive research. Furthermore, zebrafish are amenable to genetic manipulation, which has offered another important aspect for researchers studying gene effects on reproduction.

b) Other mammals: a whole industry exists with respect to semen collection and cryostorage in farm animals. Semen collection and artificial insemination have become routine procedures in livestock reproduction. Semen is usually collected by employing an artificial vagina. Sperm from desired breeding males are

cryopreserved, shipped, and used to inseminate large numbers of females. Cryopreservation of sperm, germ cells, and testicular tissue has become a standard operating procedure in the propagation of livestock species and in assisted reproduction clinics. In livestock management, strategies for cryopreservation of sperm are constantly improved (Ehmcke and Schlatt, 2008). According to difficulty of male fertility, the entire process cannot be modelled in vitro. As such, animal models, particularly mouse models, provide a valuable alternative experimentation (Jamsai and O'Bryan, 2011). Human failed fertilisation oocytes have revealed that 32.3% of human failed fertilisation oocytes contained sperm chromosomes. In contrast, if penetration occurs in failed fertilisation mouse oocytes, there is only a 1.4% rate of the spermatozoa arresting (Neuber and Powers, 2000). Bovine species can also be used as a suitable model in male fertility in humans. There are significant similarities between the human and bovine genome as well as embryonic development (Elsik et al., 2009). Contrary to human fertility research relying on anecdotal records of one or a few infertility treatment cycles per couple, there is an abundance of field fertility data available in the agricultural sector focused on improving livestock reproduction. Sperm samples from bulls with detailed breeding records for thousands of AI services exist. In addition, well-documented fertility phenotype records enhance the breadth of knowledge from animal research data (Feugang et al., 2009). Furthermore, bulls produce such vast numbers of spermatozoa that semen can be repeatedly collected from the same donor in large quantities, allowing IVF and embryo culture to be well developed, accurate, and reliable.

OBJECTIVES

The overall objective of this work was to study best techniques and methods to evaluate sperm DNA fragmentation and the impacts of oxidative stress on spermatozoa function, mainly in DNA integrity. For this last aim, zebrafish (*Danio rerio*) was used as an animal model. However, several experiments were required before specifically studying the relationship between oxidative stress and DNA fragmentation and its objective evaluation. To this end, several specific objectives were proposed:

1. To use zebrafish (*Danio rerio*) as our first model in male infertility. The first step was to establish and optimise the characteristics of sperm motility and kinetics after sperm activation in this species in different conditions of osmolality and storage methods over time (Experiment 1).

2. To study the effect of different degrees of oxidative stress generated by H_2O_2 on sperm motility and parameters and how these affected sperm DNA integrity in zebrafish. Moreover, a more suitable way to measure the degree of DNA integrity as both a dichotomous and continuous variable was studied (Experiment 2).

3. In humans, morphometric evaluations of DNA fragmentation from the SCD technique using two commercial kits were studied. Results using the mathematical clusters that provide a classification matrix of different subpopulations of sperm head DNA-reacted cells were compared (Experiment 3).

Experiment 1

Effect of activation media with different osmolality and cool storage on spermatozoa motility parameters along the time on zebrafish

S. Sadeghi *, J. Nuñez, C. Soler and M. A. Silvestre

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PROOF

RESEARCH PAPER

Effect of the Activation Media with Different Osmolality and Cool Storage on Spermatozoa Motility Parameters over Time in Zebrafish, *Danio rerio*

Sara Sadeghi*, Javier Nuñez, Carles Soler, Miguel Angel Silvestre

[1] Departamento de Biología Funcional y Antropología Física, Universitat de Valencia, Burjassot, 46100 Valencia, Spain.

* Corresponding Author: Tel.: +34.671 844000;
E-mail: sara.sadeghi1@gmail.com

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Abstract

In the present work, the effects of the activation media with different osmolality and storage conditions on sperm motility and kinetic parameters over time after activation, using CASA system (ISAS®) in Danio rerio were analysed. The effect of pooled and individual ejaculate samples was also studied. Spermatozoids activated with high osmolality medium showed higher values in motility parameters and kinematic parameters in comparison with low osmolality medium. Respect to the individual ejaculates or the pool, although significant differences were observed in some parameters, no relevant changes were observed in total motility neither in kinetic parameter. Total motility and velocity of sperm showed significant lower values after sperm storage for 24 h at 4 °C.

Keywords: Sperm motility, activation, osmolality, zebrafish, CASA, kinetic parameters

Introduction

Guaranteeing the conditions in which spermatozoa motility is optimal, is important, both in terms of *in vivo* and *in vitro* fertilizing of eggs for optimizing the biotechnology of fish reproduction (Cejko *et al.,* 2013). Sperm motility constitutes the basis for evaluating milt and controlling the ability of sperm to fertilize eggs. Bozkurt *et al.* (2006) confirmed a positive correlation between fertilization rates and spermatozoa motility. The spermatozoa of most fish species are immotile in the testis and seminal plasma. Motility is induced after the spermatozoa are released into aqueous environment during natural reproduction or in to the medium with different osmolality during artificial reproduction. There are clear relationship between seminal plasma composition, osmolality and the duration of fish sperm motility. Seminal plasma has a composition of ions, which affects spermatozoa function and motility. The osmolality of seminal plasma usually prevent sperm motility (Alavi and Cosson, 2005; Islam and Akhter, 2011). This shows the importance of using adequate osmolality to activate fish species spermatozoa. Many authors suggested that, as for several fresh water fish species, the primary signal for initiation of sperm motility in zebrafish *Danio rerio* (Hamilton, 1822), is a change in osmolality (Alavi and Cosson 2006; Wilson-Leedy *et al.*, 2009). Generally a decrease in the medium osmolality triggered sperm motility. *D. rerio* spermatozoa could be activated in hypotonic solutions at a wide range of osmolalities, ranging from 25 to 270 mOsm/kg, but the highest motility was observed at a range from 150 to 210 mOsm/kg (Jing *et al.,* 2009). Other parameters such as ion concentrations (K^+, Na^+ and Ca^{2+}), osmotic pressure, pH, and temperature and dilution rate can also affect sperm motility. As these conditions depolarize the cell membrane may affect the capacity of flagella motility (Alavi and Cosson, 2006; Morisawa and Suzuki, 1980).

Once motility of spermatozoa has been initiated, its duration is typically short, often less than one minute in fresh water species, with hypotonicity-associated cell swelling and lysis as a limiting factor (Alavi and Cosson, 2006; Wilson-Leedy *et al.,* 2009). It is worth considering that some characteristics, such as oocyte size, spermatozoa swimming distance, sperm motility duration and micropyle closing time are determinants in the procedures of application of insemination doses (Billard and Cosson, 1992). Several studies have described the effect of different factors like osmolality, ions, temperature on sperm motility initiation and duration, using CASA system, in species such as medaka *Orizias latipes* (Temminck and Schlegel, 1846), sturgeon, carp *Cyprinus carpio* or salmonids. However, there is still a lack of information in *D. rerio*. (Alavi *et al.,* 2009; Bastami *et*

al., 2009; Jing *et al.*, 2009; Wilson-Leedy *et al.*, 2009; Yang and Tiersch, 2009; Li *et al.*, 2012; Butts *et al.*, 2013; Cejko *et al.*, 2013; Dziewulska and Domagała, 2013). There are not enough studies about motility and sperm kinetics in D. *rerio*.

Zebrafish is an animal model in ecotoxicology and developmental biology for other vertebrate species (Seok *et al.*, 2008). Moreover, zebrafish could be an adequate model to other fish species. An important factor in fishery and aquaculture is the ability of preserving semen for a short or long period, using cryopreservation or short storage techniques. The effect of storage temperature on *D. rerio* sperm motility revealed a rapid decline of motility after 2 h when samples were stored at "room temperature" (about 25°C) , and suggested cooling of sperm sample at 4°C for prolonged storage (Jing *et al.*, 2009). Generally, the storage has negative effect on sperm viability and plasma membrane integrity and motility in several species (Perez-Cerezales *et al.*, 2009). Also, variability within males and the condition of semen storage are critical factors that determine the viability of sperm after short-term storage. In salmon, diluted semen stored for several days at 4ºC presented higher motility rates than undiluted semen (Trigo *et al.*, 2015). However, the spermatozoa motility and its duration can change not only among different males but also in each individual ejaculate (Bozkurt *et al.*, 2006).

The aim of this work was to study the effect of the activation media with different osmolality and cooled storage for 24 h at 4ºC of diluted semen on the spermatozoa motility parameters analysed by a computer assisted sperm analysis (CASA) over time in *D. rerio*. Moreover, the effect of pooled or individual stripping samples on sperm motility was studied.

Materials and Methods

Animals and Semen Collection

Five adult *D. rerio* were obtained from Universidad Politécnica de Valencia. Animals were kept in a recirculating system that continuously filtered the system water to maintain the water quality required for a healthy aquatic environment. The tank temperature was generally maintained between 26-28.5 °C and the lighting conditions were 14:10 h (light: dark). The animals were fed with commercial dry fish food.

Sperm was initially diluted without activation, in Hank's balanced salt solution (HBSS). The solution was composed of 1.5 g of bovine serum albumin (BSA; Sigma-Aldrich H8264) and 0.1 g NaCl dissolved in 100 mL Hank's solution (Sigma-Aldrich A7906) with an osmolality of approximately 300 mOsm/kg. The osmolality was measured by an osmometer (The Fiske® Micro-osmometer 210).

The semen collection process was done approximately after 15 minutes the light period starting. Fishes were slightly anaesthetized with natural clove oil (0.18 mL in 1 L of fresh water. Rinse in fresh water, and dried gently with paper towels, and place belly up in a slit in a damp sponge. The semen was collected with glass capillary tubes after applying gentle pressure to the abdomen, from the top to the genital pore. Each ejaculate was diluted with 75 µL of HBSS solution into an Eppendorf microtube. In this study, two groups of sperm samples were used. Sperm samples stripped from at least from 4 males and/or pooled in equal amounts. Samples were stored into a fridge at 4° C until their use. Samples used on the same day of collection were stored for less than 2 h in the fridge and they were considered as fresh diluted semen. Samples were stored for 24 h, were considered cooled diluted semen.

Computer-Assisted Sperm Analysis (CASA)

Spermatozoa motility parameters were obtained by a CASA system (ISAS® v1.2; PROISER S.L., Paterna, Spain). The microscope used was a triocular UOP equipped with a negative contrast phase objective (lens Plan 10xPHN, PROISER), and recordings were made by a digital camera (Basler, A780-54fm, Ahrensburd, Germany). Then, each recording was analysed with the following acquire and track settings: Image per second: 25; image fields max: 25; with the area surface of 10<90 µm². The following values were determined by CASA: the percentage of motile sperm (MOT,%), percentage of sperm moving with straightness (STR,%), curvilinear velocity (VCL, µm/sec), average velocity path (VAP, µm/sec), straight-line velocity (VSL, µm/sec), wobble of the curvilinear trajectory (WOB, ratio of VAP/VCL,%), linearity movement (LIN,%) and beat cross frequency (BCF, Hz).

Activation of Spermatozoa

Two sperm activation solutions with different osmolality were studied:

- Medium H composed of 9 mL of HBSA plus 3 mL of deionized water (~ 90 to 110 mOsm/kg).
- Medium L composed of fresh water (~ 15 to 20 mOsm/kg).

Sperm were activated at room temperature by mixture of 3 µL of diluted semen sample to 15 µL of activation medium (H or L) on a microslide (26x76 mm, Deltalab), using a micropipette followed by 2 seconds of gentle stirring with the micropipette tip.

Approximately 3 µL of this mixture of activated sperm was quickly applied to the sperm counter chamber (Spermtrack®, 10 µm depth; PROISER S.L., Paterna, Spain). Sperm motility was estimated in the same field and captured each 5 seconds until 2.5 minutes, then, each 30 seconds, until the 80% of spermatozoids became immotile.

Experimental Design

Study 1:The effect of two sperm activation media with different osmolality on motility and kinetic parameters of pooled fresh semen.

It was studied the effect of two activation media (H and L) on sperm motility and kinetic parameters over time in pooled fresh samples. Each pool sample obtained from five adult male. Four replicates were used.

Study 2:The effect of type of sperm sample (individual ejaculates or pooled samples) on the evolution of sperm motility and kinetics over time.

It was studied the effect of the pool *vs.* individual semen samples. For the individual group, we use an arithmetic mean of five adult males per replicate. For that, data from sperm activated with media H and L from study 1 (for pooled samples) were analysed together. Four replicates were used.

Study 3: The effect of short-term cool storage of pooled semen on sperm motility and kinetic parameters.

It was studied the effect of diluted sperm storage at 4ºC for approximately 24 h on sperm motility and kinetics over time in pooled samples, and compared with pooled fresh samples. A pool from five adult males and four replicates were used.

Statistical Analysis

All the statistical analysis was performed using SPSS software (IBM SPSS statistics V21). Results of sperm motility rate were analysed by a binary logistic GLM procedure. Results of kinetic parameters (VLC, VSL, STR, LIN, WOB, VAP and BCF) were analysed by GLM procedure following a linear model. A probability of $P<0.05$ was considered to be statistically significant. For Study 1, a model with two factors, activation media osmolality (2 levels: H or L) and video capture (20 levels; 20 consecutive video captures), and "two-way interaction" were used. For Study 2, a model with two factors, sperm sample (2 levels: I or P) and video capture (20 levels; 20 consecutive captures), and "two-way interactions" were used. For Study 3, a model with two factors, storage conditions (2 levels: fresh or cooled for 24 h at 4ºC) and video capture (16 levels; 16 consecutive captures) and "two-way interactions" were used.

Results

Effect of Osmolality of Sperm Activation Media On Motility and Kinetic Parameters of Pooled Fresh Semen

Results from the Study 1 were showed in Table 1 and Figures 1A-H. Spermatozoa activated with medium H showed significant higher total motility rate than L (75.7 ± 0.4 vs. 63.7 ± 0.6 respectively, Table 1). Respect to spermatozoa kinetics, activation media affected almost all studied parameters except WOP. Sperm activated with medium H showed higher values than medium L. The total motility x time capture interaction was statistically significant, nevertheless motility drop over time capture, were similar up to the first 7 video captures in both H and L media, (approximately 55 seconds; Figure 1A). In reference to kinetic parameters, time affected respectively VCL, VSL, VAP and BCF (Table1). For VCL, VSL VAP, and BCF, values began to decrease for L media starting from 2rd-3rd time capture (Figure 1B, 1C, 1E and 1H respectively). The LIN, STR and WOB parameters presented higher values in medium L until 6th time capture and from this moment these values were lower than in medium H (Figure 1D, 1F and 1G).

Effect of Type of Sperm Sample (Individual Ejaculates or Pooled Samples) on Sperm Motility and Kinetics Over Time

Total motility of the pooled samples was slightly higher than individual (Table 2). However, individual samples maintained the motility better than pooled samples (Figure 2A). Respect to kinetics of sperm motility, all the parameters was significantly different except BCF. Otherwise, all the velocity dependents parameters like, VCL, VSL and VAP showed an increase (Table 2, Figure 2B, 2C and 2E).

Effect of Short-Term Cool Storage on Sperm Motility and Kinetics

In 3rd study, according to the results present in Table 3, significant differences were observed between fresh and cooled stored samples in total motility rate (78.1 ± 0.45 vs. 41.4 ± 0.8 for fresh and cooled samples respectively). Respects to kinetics of spermatozoa motility, the values are lower for cool stored samples than fresh pooled samples. The interaction term between captures and storage conditions of samples was also significantly different (Figures 3A-H). The LIN, STR and BCF parameters of cooled samples presented higher values until 5th time capture, and then it showed a decrease, with lower values than fresh pool samples (Figure 3D, 3F and 3H).

Discussion

Sperm motility is a key factor to determine semen quality and clearly is related to osmolality which having important relevance in fertilizing capacity and the duration of sperm motility of freshwater fish species (Ingermann *et al.,* 2011). Fish sperm is immotile in the testis or in an osmolality similar to its blood plasma or the seminal plasma osmolality (*300*-315 mOsm/kg).

The initiation of motility can be controlled by changes in ion concentration of the external medium

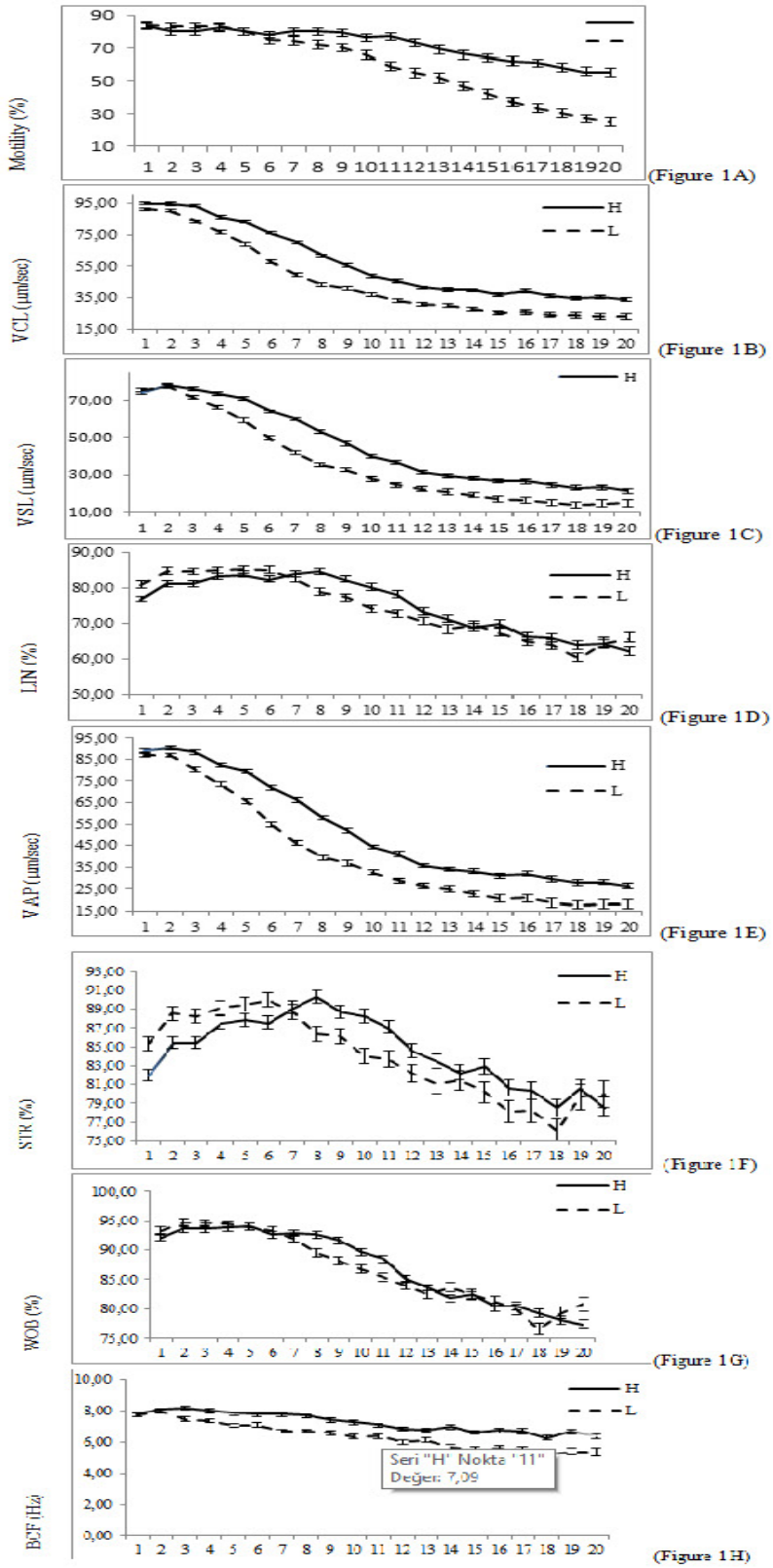


Figure 1.Effect of activation media H or L on motility and kinetic parameters during 20 captures. Motility(%) (Fig. 1A), VCL: curvilinear velocity (µm/sec)(Fig 1B), VSL: straight-line velocity (µm/sec)(Fig 1C), LIN: linearity of the curvilinear trajectory (%)(Fig 1D), VAP: average path velocity (µm/sec)(Fig 1E), STR: straightness (%)(Fig 1F), WOB: wobble (VAP/VCL)(%)(Fig 1G), BCF: beat-cross frequency (Hz)(Fig 1H), from sperm parameters as measured at different video capture rate (20 seconds post activation) each capture presented 5 seconds.

(Alavi and Cosson, 2005, 2006). Hypotonic medium initiates the motility of freshwater fish spermatozoa such as common carp *Cyprinus carpio* and goldfish *Carassius auratus auratus* (L). In salmonids (Cuvier, 1816) and sturgeon *Acipenser sturio* (Linnaeus, 1758), the decrease in K^+ concentration upon dilution is a key factor for sperm initiation, whereas in the cyprinids, a decrease in osmolality is the basis of this sperm activation (Islam and Akhter, 2011; Reinardy *et al.,* 2013).

In experiment 1, sperm motility rate was greater and duration was prolonged in time with the activation medium with the highest osmolality. To the best of our knowledge there is still lack of information focused on the effect of osmolality of sperm activation media on total motility and kinetic parameters evaluated by CASA in *D. rerio*. Jing *et al.* (2009) indicated that the osmolality of activation medium is the first signal for sperm motility, by using two different activation solutions (ionic and ion-free). However, they assessed sperm motility by subjective visual estimation, and no data showed about sperm kinetics.

Tejerina *et al.* (2008) discussed that sperm motility parameters analysed using CASA system provided a more objective assessment of sperm motility in mammals. CASA technology has several advantages as compared to manual counting, such as, fast analysis; increase consistence when the same setting is used, and high statistical power due to objective analysis of numerous sperm (Schleh and Leoni, 2013; Lammers *et al.,* 2014). Unlike Jing and collaborators' work, we used CASA system for evaluating the sperm motility, contributing with more objective and additional data such as kinetic parameters. Moreover, we studied the effect of different ejaculates or pooled samples. Rurengwa *et al.* (2004) stablished with the CASA system, that the progressive motility velocities correlated better with fertilization rates than the other parameters. Recently, using CASA system, Wilson-Leedy *et al.* (2009) studied the effect of several osmolality ranges on *D. rerio* sperm motility and observed that moderate NaCl concentration (80 mM) remained the highest sperm motility rate, but not with low or high NaCl concentrations (0 or 120 mM). In addition to osmolality, the pH and temperature are also important factors that involved with sperm motility in fresh water species (Alavi and Cosson, 2005). In other freshwater species, the motility activation may also depend on osmolality rather than extracellular ions concentration (*Prochilodus lineatus* and *Brycon orbignyanus*, medaka *Orizias latipes*, medaka *Orizias latipes)* (Yang and Tiersch, 2009).

On the other hand, curvilinear velocity (VCL) was not affected by osmotic concentration of the activation medium, and velocity has a better response to intermediate osmotic concentration (Wilson-leedy *et al.,* 2009). The linearity movement (LIN) and straightness of swimming (STR) did not show a relevant effect by activation media with different osmolality. However, values of VCL, VSL, and VAP showed a significant increase in high osmolality medium. Cejko *et al.* (2013) showed that carp *Cyprinus carpio* sperm activation in distilled water resulted in a decrease in not only the motility parameter but also the VSL and BCF compared with a medium with high osmolality. In contrast with our

Table 1. The effect of two sperm activation media with different osmolality on motility and kinetic parameters of pooled fresh semen along time

	Total Motility Rate	VCL	VSL	LIN	VAP	STR	WOP	BCF
Medium H	75.7 ± 0.4^a	57.3 ± 0.2^a	45.4 ± 0.2^a	75.2 ± 0.2^a	52.0 ± 0.2^a	84.6 ± 0.2^a	87.1 ± 0.1	7.2 ± 0.0^a
Medium L	63.7 ± 0.6^b	45.1 ± 0.3^b	35.8 ± 0.3^b	74.3 ± 0.3^b	40.9 ± 0.3^b	$83.8\ 0.2^b$	83.8 ± 0.2	6.4 ± 0.0^b

[1] Medium H was composed by 9 mL of HBSS plus 3 mL of deionized water (~ 90 to 110 mOsm/Kg); Medium L composed by aged water (~ 15 to 20 mOsm/Kg). Values are expressed as the means ± SEM. VCL: curvilinear velocity, VSL: straight-line velocity, LIN: linearity of the curvilinear trajectory, VAP: average path velocity, STR: straightness, BCF: beat-cross frequency WOB: wobble (VAP/VCL). ^{ab}Numbers within columns with different superscripts differ ($P<0.05$)

Table 2. The Effect of type of sperm sample (individual ejaculates or pooled samples) on the evolution of sperm motility and kinetics over time.

Group[2]	Total Motility Rate	VCL	VSL	LIN	VAP	STR	WOP	BCF
Individual	65.2 ± 0.2^b	62.8 ± 0.2^a	48.1 ± 0.2^a	75.0 ± 0.1^b	57.6 ± 0.2^a	82.4 ± 0.1^b	89.4 ± 0.1^a	6.8 ± 0.0^a
Pool	67.3 ± 0.4^a	55.7 ± 0.3^b	45.5 ± 0.3^b	77.8 ± 0.2^a	51.5 ± 0.3^b	85.8 ± 0.2^a	89.1 ± 0.1^b	6.9 ± 0.0^b

[2] Values are expressed as the means ± SEM. VCL: curvilinear velocity, VSL: straight-line velocity, LIN: linearity of the curvilinear trajectory, VAP: average path velocity, STR: straightness, BCF: beat-cross frequency WOB: wobble (VAP/VCL). ^{ab}Numbers within columns with different superscripts differ ($P<0.05$)

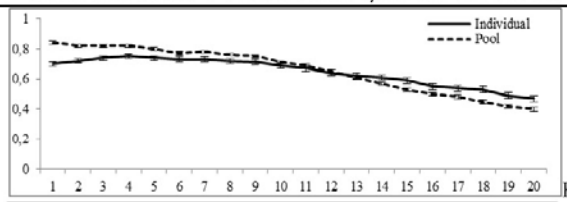


Figure 2A

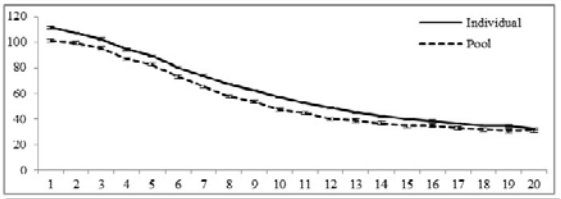


Figure 2B

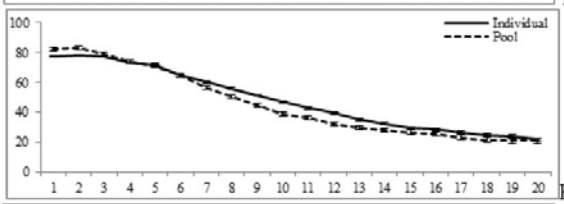


Figure 2C

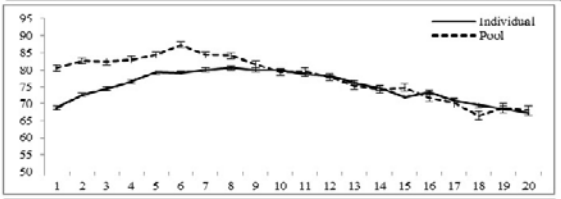


Figure 2D

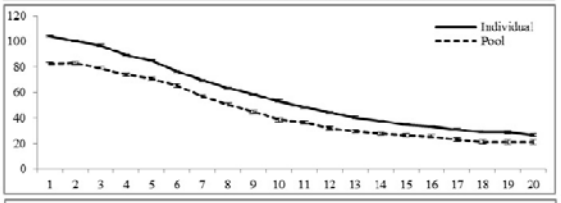


Figure 2E

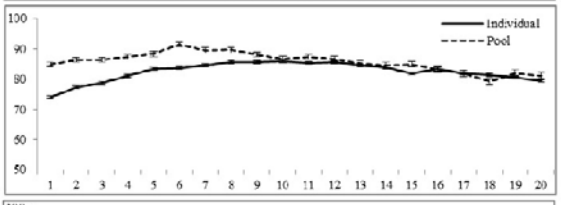


Figure 2F

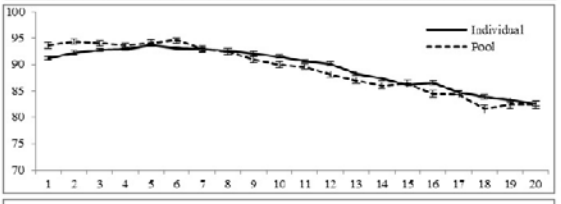


Figure 2G

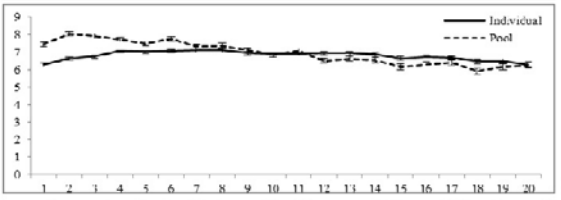


Figure 2H

Figure 2. Effect of type of samples (Individual vs. Pool) on motility and kinetics of spermatozoa during 20 captures. Motility (%) (Fig. 1A), VCL: curvilinear velocity (µm/sec)(Fig 1B), VSL: straight-line velocity (µm/sec)(Fig 1C), LIN: linearity of the curvilinear trajectory (%)(Fig 1D), VAP: average path velocity (µm/sec)(Fig 1E), STR: straightness (%)(Fig 1F), WOB: wobble (VAP/VCL)(%)(Fig 1G), BCF: beat-cross frequency (Hz)(Fig 1H), from sperm parameters as measured at different video capture rate (20 seconds post activation) each capture presented 5 seconds.

results, Wilson-Leedy *et al.* (2009) concluded that, initial velocity (VCL) was insensitive to osmolality of activation medium, but velocities were better maintained over time by activation media with intermediate osmotic concentrations in *D. rerio*. Butts *et al.* (2013) demonstrated higher osmolality in activation medium, higher velocity (curvilinear) in redside dace *Clinostomus elongates* spermatozoa for the first 10 s. Cosson (2010) linked a higher initial velocity with lower duration of sperm motility but in our case, we observed a higher initial velocity in high osmolality medium was maintained with longer swimming period and total duration of motility in VCL parameter. The reason to explain these results could is the quantity of stored ATP. In this sense, it is known that the primary source that supports spermatozoa motility in the quantity of stored ATP in sperm cells (Ingermann *et al.,* 2011). It also has been reported that the ATP content was lower at low osmolality medium at the end of the carp sperm motility (Billard *et al.,* 1995). In carp, a decrease in MOT, VCL, and BCF values was associated with using up the intracellular ATP supplies (Perchec *et al.,* 1995). Moreover, the decrease in sperm motility duration in low osmolality medium may be caused by damage of the flagellum after plasma membrane alteration (Alavi *et al., 2009)*.

In many industrial and laboratory hatcheries large volumes of eggs were fertilized with small volumes of semen, which frequently originates from a small potentially ejaculation volume (Targonska *et al.,* 2008). Also, it was suggested that an option in order to decrease variability of *D. rerio* in vitro fertilization efficiency could be pooling gametes (Hagedorn and Carter, 2011) as it accrued in some mammals or avian livestock species mentioned that pooling of gametes from different individual might be contemplated an option to decrease variability in fertility (Donoghue *et al.,* 2003). Moreover, there was no effect on fertilization success using pooled male frozen/thawed sperm with individual female eggs in *D. rerio* (Hagedorn and Carter, 2011). Targonska *et al.* (2008) indicated that the use of the pooled semen obtained from many males, allowed obtaining better breeding results, because of the fertilization success of some males was significantly higher than the others. In the second experiment, the effect of each kind of sample, individual ejaculates or pooled semen from different males on total motility and kinetic parameters was studied. There are no previous studies about how the sort of sample (individual vs. pool semen samples) can be affecting the spermatozoa different motility parameters in *D.* rerio. We observed statistical differences between individual ejaculates or pooled samples, although these differences were not very relevant. Hagedorn and Carter (2011) mentioned the effect of pooling gametes (female and male) in fertilization rates, observing that the pooling sperm samples did not affect fertilization rates. In mammals, Batista *et al.* (2012) observed that differences in sperm motility between individual and pooled samples were evident after 8 h of preservation and depending of semen storage temperature. In the present work, the participation of males to the pool was not exactly the same since it was very difficult to adjust male contribution due to the sample size was smaller in comparison with other animal species. In this sense, the difference in sperm motility, were due to different male contribution to the pool. Other possibility could be due to the effect of the semen sample of the same ejaculate.

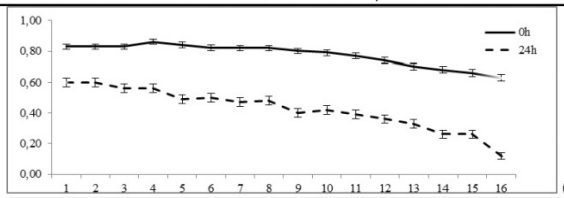
In the last study, we observed that cooled storage affected motility and kinetics in *D. rerio* sperm. We observed a higher sperm motility rates for the fresh semen samples, and the drop of total sperm motility over time seems more pronounced in the sperm samples stored at 4ºC. The effective short-term storage of semen is essential when processing multiple sperm samples use and semen must be transported far away to the collection sites, this make it possible to maintain viable spermatozoa for a short time (Bozkurt *et al.,* 2009; Aramli *et al.,* 2013; Trigo *et al.,* 2015). However, there is no data concerning analysis effect of cool storage (24 h - 4ºC) using CASA in D. *rerio*. Sahin *et al.* (2013) reported that sperm motility in rainbow trout *Oncorhynchus mykiss* (Walbaum, 1792) was affected during preservation and motile cells decreased as the length of storage. Short storage (12-36 h) in 4ºC could be best period and temperature for cool short storage. Cardona-Costa *et al.* (2009) observed the same rate of larvae at 24 h after in vitro fertilization, using *D. rerio* sperm stored at 8ºC for 24 h in comparison with fresh sperm. They also observed the optimal motility after 48-72 h in some cases.

Metabolic activity of sperm decreases during cooling storage of sperm samples in mammals, being possible to prolong their lifespan (Aurich, 2008). One explanation could be that there is decreasing trend in the intracellular ATP concentration during the storage

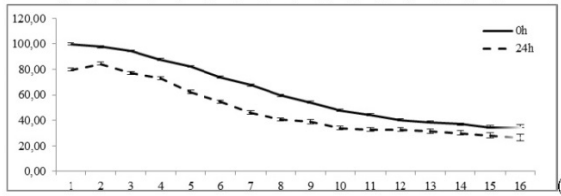
Table 3-The Effect of short-term and cool storage on sperm motility and kinetic parameters.

Group[3]	Total Motility Rate	VCL	VSL	LIN	VAP	STR	WOP	BCF
0h	78.1 ± 0.5^{a}	62.2 ± 0.2^{a}	50.5 ± 0.2^{a}	78.0 ± 0.2^{a}	57.6 ± 0.2^{a}	85.5 ± 0.2^{a}	89.6 ± 0.1^{a}	7.2 ± 0.0^{a}
24h	41.4 ± 0.8^{b}	48.3 ± 0.4^{b}	37.8 ± 0.4^{b}	72.4 ± 0.3^{b}	42.9 ± 0.4^{b}	83.0 ± 0.2^{b}	84.9 ± 0.2^{b}	7.0 ± 0.0^{b}

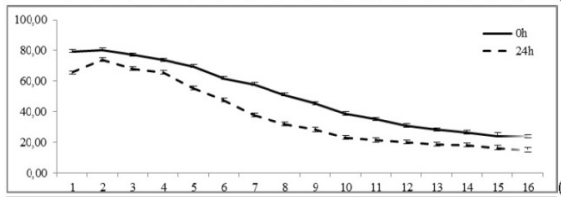
[3] The pool samples were stored at 4°C for 24 hours as the group of (24 h). Values are expressed as the means ± SEM. VCL: curvilinear velocity, VSL: straight-line velocity, LIN: linearity of the curvilinear trajectory, VAP: average path velocity, STR: straightness, BCF: beat-cross frequency WOB: wobble (VAP/VCL). ^{ab}Numbers within columns with different superscripts differ (P<0.05)



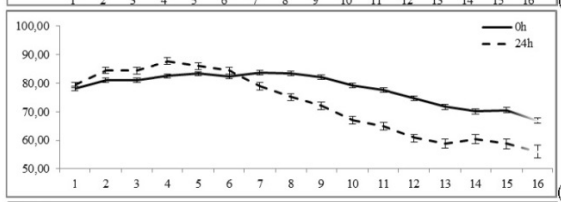
(Figure 3A)



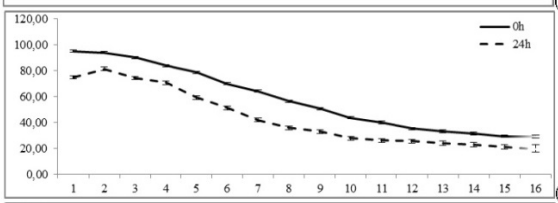
(Figure 3B)



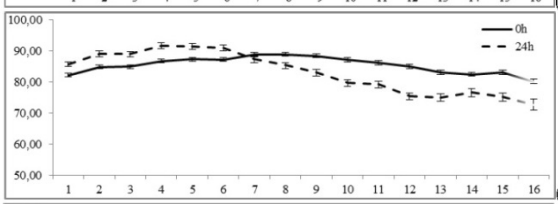
(Figure 3C)



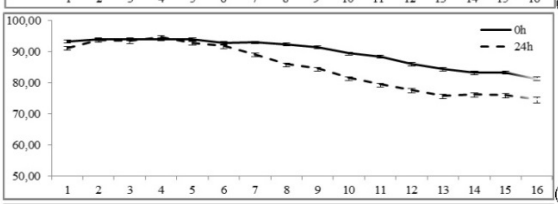
(Figure 3D)



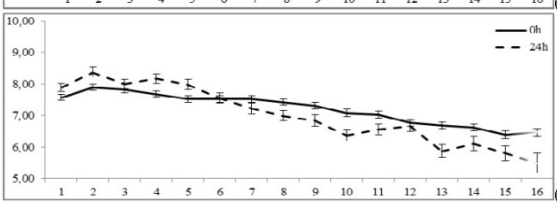
(Figure 3E)



(Figure 3F)



(Figure 3G)



(Figure 3H)

Figure 3. Effect of storage time on motility and kinetics along 16 captures (24 hours at 4°C). Motility (%) (Fig. 1A), VCL: curvilinear velocity (µm/sec)(Fig 1B), VSL: straight-line velocity (µm/sec)(Fig 1C), LIN: linearity of the curvilinear trajectory (%)(Fig 1D), VAP: average path velocity (µm/sec)(Fig 1E), STR: straightness (%)(Fig 1F), WOB: wobble (VAP/VCL)(%)(Fig 1G), BCF: beat-cross frequency (Hz)(Fig 1H), from sperm parameters as measured at different video capture rate (16 seconds post activation) each capture presented 5 seconds.

period in salmonid semen (Trigo *et al.,* 2015). The declines in ATP contents and oxidative stress can cause metabolic or functional disorders, and reducing spermatozoa motility (Aramli *et al.,* 2013). In addition, the alteration of sperm motility after short cool storage may be caused by insufficient oxygen supply occurring during storage. In rainbow trout, the decrease of sperm motility and VSL, LIN was more pronounced than the other parameters (Dietrich *et al.,* 2005).

In the present work the steady decrease in motility duration and other kinetic parameters except from LIN, STR and BCF after 24 h storage time was observed. The decrease in sperm ATP content it could be correlated with affected parameters.

In summary, spermatozoids activated with medium with a high osmolality medium showed higher values in motility and kinetic parameters in comparison with low osmolality medium. Respect to the individual ejaculates or the pool, although significant differences were observed in some parameters, no relevant changes were observed in total motility neither in kinetic parameter. Total motility and velocity of sperm showed significant lower values after cool storage at 4ºC for 24 h.

Acknowledgments

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Experiment 2

Effect of the different oxidative stress degrees generated by hydrogen peroxide on motility and DNA fragmentation of zebrafish (*Danio rerio*)

Sara Sadeghi, José. Pertusa, Jesús Yaniz, Javier. Nuñez, Carles. Soler, Miguel Angel Silvestre*

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ORIGINAL ARTICLE

WILEY Reproduction in Domestic Animals

Effect of different oxidative stress degrees generated by hydrogen peroxide on motility and DNA fragmentation of zebrafish (*Danio rerio*) spermatozoa

Sara Sadeghi[1] | José Pertusa[1] | Jesús L. Yaniz[2] | Javier Nuñez[1] | Carles Soler[1] | Miguel Angel Silvestre[1]

[1]Departament de Biologia Cellular, Biologia Funcional i Antropologia Fisica. Universitat de València, Burjassot, Spain

[2]TECNOGAM research group, Environmental Sciences Institute (IUCA), University of Zaragoza, Huesca, Spain

Correspondence
Miguel Angel Silvestre, Departamento de Biología Celular, Biología Funcional y Antropología Física; Universitat de València. 46100 Burjassot, Valencia, Spain.
Email: miguel.silvestre@uv.es

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Abstract

An increase in reactive oxygen species (ROS) or decrease in antioxidant barriers can provoke lipid peroxidation of the membranes or DNA damage of the spermatozoa. The aim of this work is to study the effect of the different degrees of oxidative stress generated by H_2O_2 incubation on total motility, kinetics, and DNA fragmentation of zebrafish (*Danio rerio*) spermatozoa. For this process, experimental groups were incubated in 50 μM (Low; L) and 200 μM (High; H) H_2O_2, respectively, for 20 min at 4°C. Sperm motility parameters were obtained with a computer-assisted sperm analysis (CASA) system. Sperm DNA fragmentation (SDF) was assessed using the sperm chromatin dispersion test. Both low and high H_2O_2 concentration groups showed lower motility than control groups. Progressive motility of spermatozoa incubated in the H group dropped rapidly in comparison with other groups. Regarding SDF, the control and L groups had significantly lower values than the H group (25.0% and 31.6% vs. 48.1% fragmented sperm for C, L, and H groups, respectively; $p < 0.05$). Sperm motility, mostly progressive motility, decreased as H_2O_2 concentration increased, mainly when time after sperm activation increased. SDF increased as the H_2O_2 concentration increased. However, measurements of the halo area did not agree with the subjective SDF rate.

KEYWORDS
CASA-mot, DNA fragmentation, hydrogen peroxide, sperm chromatin dispersion, zebrafish

1 | INTRODUCTION

Spermatozoa intrinsically produce reactive oxygen species (ROS), with mitochondria metabolism as the main producer. At physiological concentrations, ROS occupy an essential role in mammal sperm capacitation (Aitken, 2006). However, a disproportionate increase in ROS or decrease in antioxidant barriers can disrupt the equilibrium, producing a redox imbalance or oxidative stress, which generates damage as lipid peroxidation of the membranes or DNA damage of the spermatozoa (Aitken, 2006; Bui, Sharma, Henkel, & Agarwal, 2018; Lopes, Jurisicova, Sun, & Casper, 1998; Pereira, Sá, Barros, & Sousa, 2015; Walczak-Jedrzejowska, Wolski, & Slowikowska-Hilczer, 2013). A high level of ROS is strongly associated with human male factor infertility (Agarwal et al., 2006). In human, an overproduction of ROS is due to immature spermatozoa and leucocytes in the seminal plasma, and their number can increase with inflammation, infection and cellular defense mechanisms (Henkel, 2011). A great number of factors can contribute to increase oxidative stress, such as genitourinary infections, varicocele, stress, and smoking among others (Bui et al., 2018).

On the other hand, sperm treatments, such as extender dilution or cryopreservation, may increase the generation of additional ROS

(Figueroa, Valdebenito, & Farias, 2016; Walczak-Jedrzejowska et al., 2013). Hydrogen peroxide (H_2O_2) is one of the compounds of ROS that is made in sperm. Oxidative stress produces DNA damage, including DNA fragmentation, mitochondrial DNA damage, epigenetic abnormalities, telomere attrition, and Y chromosome microdeletions (Bui et al., 2018). The incubation of sperm in low H_2O_2 concentrations was found to significantly increase damage of zebrafish (*Danio rerio*) sperm DNA (Reinardy, Skippins, Henry, & Jha, 2013). Nevertheless, the influence of H_2O_2 on zebrafish sperm motility was not addressed in that study. Other studies have explored the effect of oxidative stress by the xanthine system on sperm motility (by subjective evaluation) and membrane integrity, but not DNA fragmentation (Hagedorn, McCarthy, Carter, & Meyers, 2012). However, motility assessment by the computer-assisted sperm analysis (CASA) system (more objective) and their evolution through time (Sadeghi, Nuñez, Soler, & Silvestre, 2017) could provide more interesting data. In rainbow trout (*Oncorhynchus mykiss)* (Walbaum 1792), Dietrich et al. (2005) observed that exposing sperm to a low concentration of H_2O_2 (1 mM) did not significant affect total sperm motility (only the VCL parameter was affected), although DNA integrity was negatively affected. On the contrary, H_2O_2 concentrations higher than 1 mM of H_2O_2 reduced sperm motility (Dietrich et al., 2005). In this way, a low concentration of the oxidative factor could affect the viability of sperm without a detectable decline in total sperm motility. We have not found existing information about the effect of H_2O_2 incubation on total motility and kinetic parameters measured by CASA system, and DNA fragmentation of zebrafish sperm.

Furthermore, the assessment of sperm DNA fragmentation (SDF) has been addressed by several methodologies ranging from complex or expensive tests to quite simple ones (Evenson, 2016). In this study, we used the sperm chromatin dispersion test (SCDT) as it does not require a flow cytometer or fluorescence microscopy. In the majority of SCDTs, DNA fragmentation was evaluated as a dichotomous variable, either fragmented or not, by an operator. In this study, in addition to motility evaluation with CASA systems, we attempted a more objective evaluation of DNA fragmentation by measuring it as an area of the sperm halo.

With the above information in mind, the aim of this work was to study the effect of the different degrees of oxidative stress generated by H_2O_2 incubation on total motility, kinetics, and DNA fragmentation of zebrafish spermatozoa. Moreover, we evaluated DNA fragmentation both as a dichotomous and continuous variable.

2 | MATERIAL AND METHODS

2.1 | Animals and semen collection

Adult specimens obtained from a local pet shop were used. Animals were kept in aquariums at the Central Service for Experimental Research of the Universitat de València. In brief, fish were kept in a recirculating system that continuously filtered water to maintain the quality required for a healthy aquatic environment. The tank temperature was generally maintained between 26–28.5°C, and the lighting conditions were 14:10 hr (light:dark). The semen collection process was executed approximately 15 min after the lights were turned on. Sperm must be collected on anesthetized animals. Fish were slightly anaesthetized with natural clove oil (0.18 ml/L) for less than one minute. After that, fish were washed in freshwater and dried gently with paper towels. The area around the cloaca of the fish should be dried to prevent activation of the sperm by water. To express the sperm, gentle pressure was applied on the sides of the fish in the urogenital region using plastic flat forceps or the thumb and forefinger.

Ejaculate was collected in a capillary tube that was placed at the urogenital opening as it was expelled using an embolus. Each ejaculate was approximately around 1 µl. Sperm samples from males (ranging from 7 to 9 individual fish) were pooled and then diluted

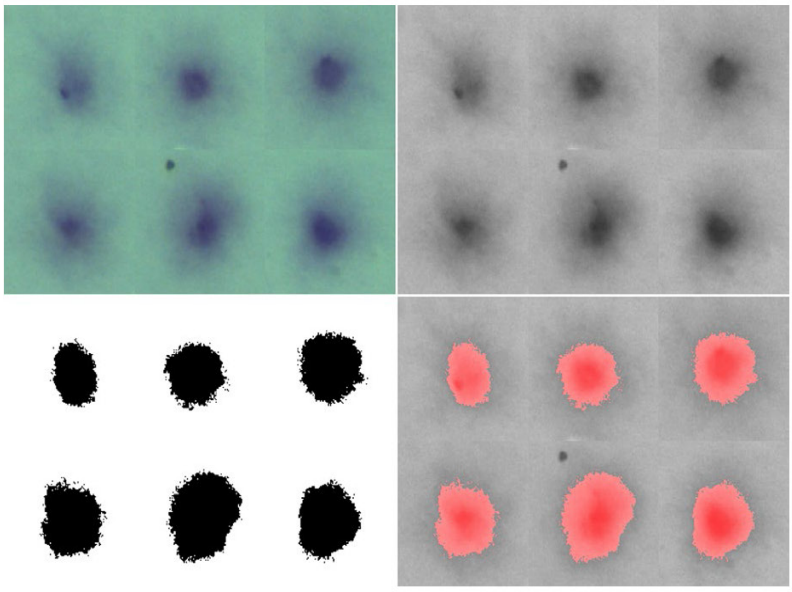

FIGURE 1 Example of sperm halo capture after applying segmentation method

in 100 µl of a solution (HBSS) composed of 1.5 g of bovine serum albumin (BSA; Sigma-Aldrich, H8264) and 0.1 g NaCl dissolved in 100 ml of Hank's solution (Sigma-Aldrich A7906); HBSS had an osmolality of approximately 300 mOsm/kg. Next, the pooled semen was split into four parts, which acted as the four experimental groups: Control 0 hr (C0), Control (C), Low (L), and High (H). The C0 group was analysed immediately after semen collection. The C group was incubated for 20 min in 4°C without H_2O_2. The L and H group samples were incubated in 50 and 200 µM H_2O_2 (Reinardy et al., 2013), respectively, for 20 min at 4°C (Dietrich et al., 2005). After incubation, cells were either processed for motility assessment as detailed below or centrifuged (8,000 *g*; 4 min). In the last case, the pellet was removed and cells were re-suspended in 15 µl of HBSS solution and frozen at −80°C until processed for the DNA fragmentation test. The semen collection procedure was approved by the Ethics and Animal Welfare Committee of Universitat de Valencia (2013/019/UVEG/016).

2.2 | Sperm motility assessment by computer-assisted sperm analysis

Spermatozoa were activated by a mixture of 3 µl of the diluted semen sample to 15 µl of deionized water. Immediately, sperm motility was measured in the same field for 90 s in seven consecutive captures (Time). Spermatozoa motility parameters were obtained by a CASA system (ISAS® v1.2; PROISER S.L., Paterna, Spain). The microscope used was a triocular UOP equipped with a negative contrast phase objective (lens Plan 10× PHN), and recordings were made with a digital camera (Basler, A780-54fm). Therefore, each recording was analysed with the following acquire and track settings: frames per second: 25; image fields max: 25; and area superficies of 10 < 90 (μm^2). The following values were determined by CASA: the percentage of total and progressive motile sperm (%), percentage of sperm moving with straightness (STR, %), curvilinear velocity (VCL, µm/s), average velocity path (VAP, µm/s), straight-line velocity (VSL, µm/s), wobble of the curvilinear trajectory (WOB, ratio of VAP/VCL, %), movement linearity (LIN, %), lateral head displacement (ALH, µm), and beat cross frequency (BCF, Hz).

2.3 | Assessment of sperm DNA fragmentation

The SDF was assessed using the SCDT with the Sperm-Halomax commercial assay (Halotech DNA SL, Madrid, Spain) designed for fish sperm (Gosálvez, López-Fernández, Hermoso, Fernández, & Kjelland, 2014). Frozen samples were thawed at room temperature to perform the SDF test. Then, 25 µl of each semen sample was mixed with 50 µl of liquefied agarose at 37°C. Next, 2 µl of the mixture was extended into marked wells, covered with a 24 × 24 mm coverslip, and placed on a cold metallic surface in the refrigerator at 4°C for 5 min. After that, the coverslips were removed and each slide was placed horizontally in 10 ml of lysing solution (Sperm-Halomax® kit) for 5 min, washed in distilled water, dehydrated by flooding with 70% and 100% ethanol (each for 2 min), drained, and allowed to dry. Sperm was stained for bright-field microscopy evaluation. Once the slides were dry, they were stained using Diff-Quick staining set (Medion Diagnostics). For this procedure, each slide was first dipped in Diff-Quick stain solution I (xanthene dye) for 7 min and then in Diff-Quick solution II (thiazine dye) for another 7 min. Thereafter, samples were washed with distilled water and allowed to dry to capture the images. The SDF was evaluated by the same operator in a subjective fashion (fragmented or not) as a dichotomous variable or by measuring the surrounding area as a continuous variable. Regarding the dichotomous variable, we considered only spermatozoa with a large clear halo as a fragmented sperm, not an

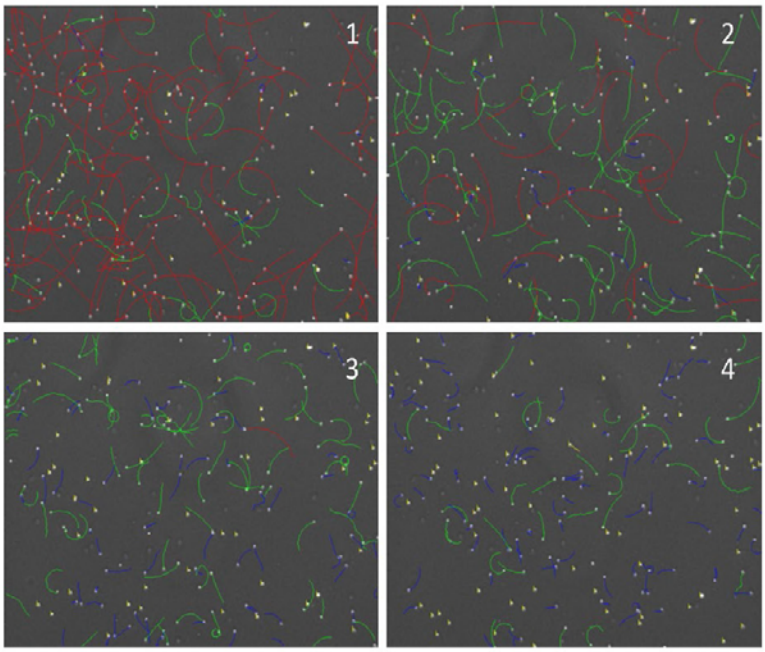

FIGURE 2 Sequence of video captures of zebrafish sperm motility of control group at the first 4 times. Sperm speeds: rapid (red), medium (green), low (blue) and static (yellow)

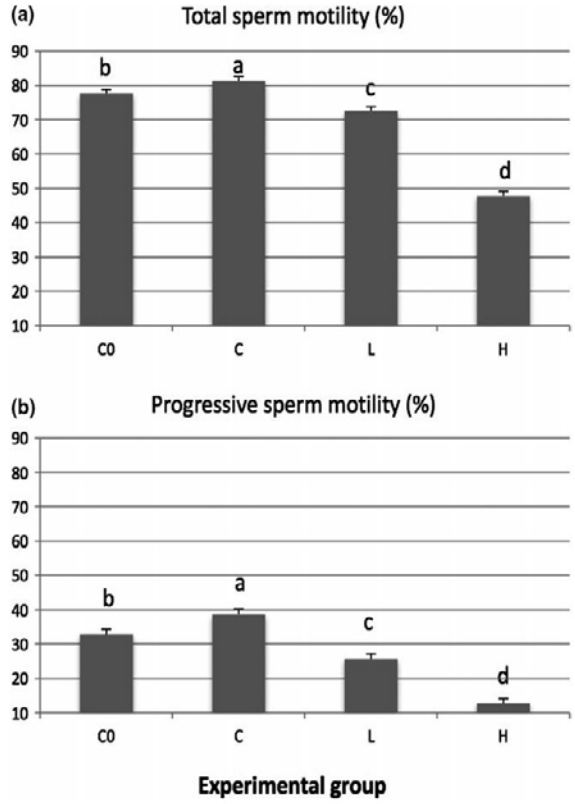

FIGURE 3 The effect of H_2O_2 on the percentage of total (a) and progressive (b) sperm motility. Experimental groups: Control 0 hr (C0), Control (C), Low (L), and High (H). The C0 group was analysed immediately after semen collection. The C group was incubated for 20 min in 4°C without H_2O_2. The L and H group samples were incubated in 50 and 200 μM H_2O_2, respectively, for 20 min at 4°C. Different superscripts (a–d) in each column within the same figure indicate differences in pairwise comparisons ($p < 0.05$)

intermediate halo (Alkmin et al., 2013; Olaciregui, Luño, Gonzalez, De, & Gil, 2015). The ImageJ software (https://imagej.nih.gov/ij/index.html) with a plug-in created for this purpose was used for measuring sperm head areas and their main diameters (ferret diameters). The segmentation method used for this image analysis was threshold selection. To select the threshold that delimits the contour of the halo, the criterion of contour selection must be performed (Figure 1). For this, the inflection point of a plotted line between the value of the background and the inside of sperm head was required. The segmentation method based on threshold selection was performed for each experimental batch to avoid batch bias. At least 250 spermatozoa per sample were analysed (ranging from 253 to 283).

2.4 | Statistical analyses

Five and six replicates were performed for sperm motility and DNA fragmentation, respectively. For motility parameters, a model with three factors (replicate, experimental group, and time) and one interaction (experimental group x time) was used. Variables for each field were calculated and used as main data. The results of the variables were analysed using a multifactor ANOVA (IBM SPSS Statistics, v19). For SDF, a logistic regression test was performed to analyse the dichotomous variable (fragmented DNA or not), and General Linear Model tests were performed for continuous variables (pixels of halo area; IBM SPSS Statistics, v19). A probability of $p < 0.05$ was considered to be statistically different.

3 | RESULTS

From statistical model, both factors (replicate, experimental group and time) and interaction had a significant effect on sperm motility. The effects of oxidative stress on total sperm motility are shown in Figures 3 and 4. Then, sperm motility significantly decreased as time increased, but this descent was different depending on experimental group ($p < 0.05$; Figure 4). Both low and high H_2O_2 concentration groups showed lower motility than the control groups ($p < 0.05$; Figure 3a). In the first measure of motility, no difference was detected among experimental groups. However, the H group motility significant decreased in comparison with other groups (Figure 4a). The L group motility was similar to the control groups until the fifth measure when it dropped significantly. The effects of oxidative stress on progressive sperm motility are shown in Figures 2 and 3. Similar to total motility, the L and H groups exhibited lower progressive sperm motility than the control groups ($p < 0.05$; Figure 3b). Progressive motility of spermatozoa incubated in the H group dropped rapidly in comparison with other groups (Figure 4b).

Concerning kinetic sperm parameters, we observed that incubating sperm for 20 min at 4°C had no effect on sperm velocities (VCL, VSL and VAP), ALH, or BCF (C0 vs. C, Table 1). However, sperm incubation in both low and high H_2O_2 concentrations reduced sperm velocities, ALH, or BCF parameters. Only the H group reduced LIN of spermatozoa ($p < 0.05$). Regarding the STR parameter, no difference was observed between the control and L groups, but the H group had a significantly high value of this parameter ($p < 0.05$).

When SDF was evaluated as a dichotomous variable, the C and L groups had significant lower values than the H group (25.0% and 31.6% vs. 48.1% fragmented sperm for the C, L, and H groups, respectively; $p < 0.05$; Table 2). Spermatozoa of the L group showed higher rates of SDF than those of the C group, but they did not reach statistical differences. Concerning the area of halos, the L group showed the smallest halo area ($p < 0.05$; Table 2). The halo areas of fragmented and nonfragmented spermatozoa were measured, and no significant differences were found between groups (Figure 5).

4 | DISCUSSION

Spermatozoa are highly specialized cells with compacted DNA that are considered transcriptionally inert (Casas & Vavouri, 2014; Ward & Coffey, 1991). Sperm cells contain a reduced cytoplasm with few antioxidants, and their plasmatic membrane is rich in unsaturated free acids and very susceptible to peroxidation (Aitken & Fisher, 1994; Hagedorn et al., 2012). Despite xanthine-xanthine

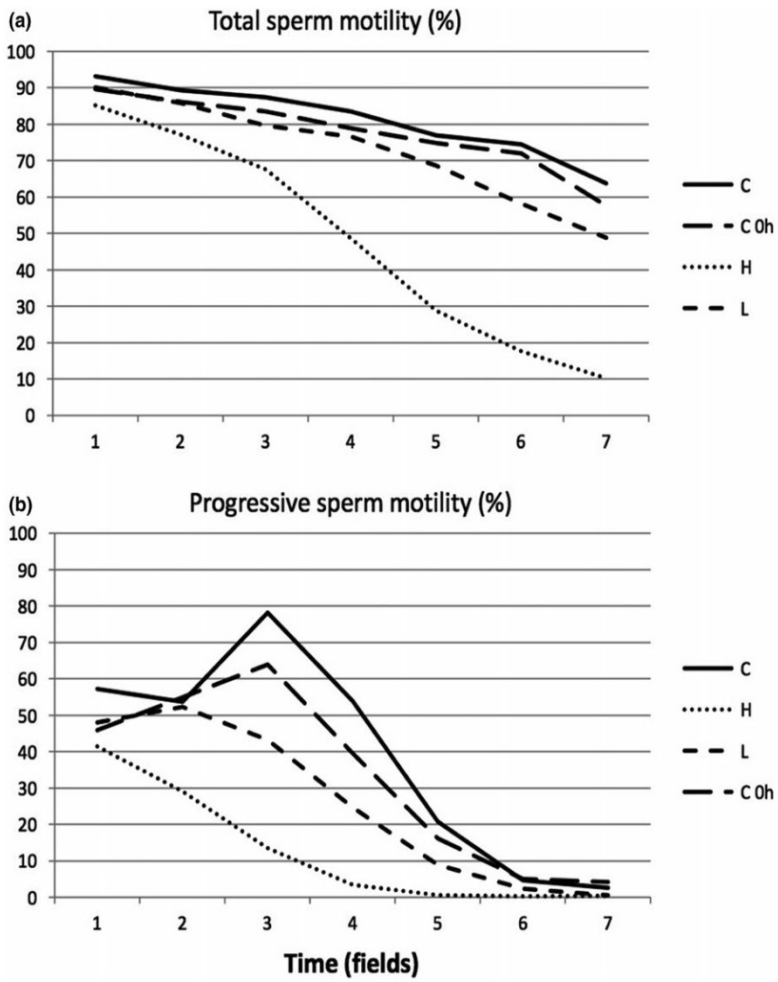

FIGURE 4 Effect of the time (fields) on the percentage of total (a) and progressive (b) sperm motility. Experimental groups: Control 0 hr (C0), Control (C), Low (L), and High (H). The C0 group was analysed immediately after semen collection. The C group was incubated for 20 min in 4°C without H_2O_2. The L and H group samples were incubated in 50 and 200 μM H_2O_2, respectively, for 20 min at 4°C

TABLE 1 Effect of different H_2O_2 concentrations on zebrafish sperm kinetics

Group[a]	VCL	VSL	VAP	LIN	STR	WOB	ALH	BCF
C0	65.7 ± 0.5^a	49.5 ± 0.5^a	61.4 ± 0.5^a	75.3 ± 0.4^a	82.7 ± 1.0^c	91.5 ± 0.2^a	1.42 ± 0.01^a	6.61 ± 0.04^a
C	66.1 ± 0.4^a	50.0 ± 0.5^a	61.2 ± 0.4^a	75.5 ± 0.3^a	87.2 ± 0.9^b	90.6 ± 0.2^b	1.44 ± 0.01^a	6.55 ± 0.04^a
L	57.3 ± 0.5^b	44.0 ± 0.5^b	53.4 ± 0.5^b	75.3 ± 0.4^a	84.0 ± 1.1^c	90.4 ± 0.2^b	1.34 ± 0.01^b	6.40 ± 0.05^b
H	38.3 ± 0.9^c	27.3 ± 1.0^c	29.9 ± 0.9^c	68.9 ± 0.7^b	117.4 ± 2.0^a	77.9 ± 0.4^c	1.24 ± 0.02^c	5.74 ± 0.09^c

Note. ALH: lateral head displacement; BCF: beat cross frequency; LIN: linearity; STR: straightness; VAP: average velocity path; VCL: curvilinear velocity; VSL: straight-line velocity; WOB: wobble of the curvilinear trajectory.

[a]Control 0 hr (C0), Control (C), Low (L) and High (H). The C0 group was analysed immediately after semen collection. The C group was incubated for 20 min in 4°C without H_2O_2. The L and H group samples were incubated in 50 and 200 μM H_2O_2 per ml, respectively, for 20 min at 4°C. Different superscripts (a–c) within the same column indicate differences from pairwise comparisons ($p < 0.05$).

oxidase system, generating a mixture of O_2^- and H_2O_2 (Aitken, Buckingham, & Harkiss, 1993) did not reduce the membrane integrity of zebrafish sperm (Hagedorn et al., 2012). Thus, spermatozoa are more susceptible to oxidative stress than somatic cells, as spermatozoa do not have the same capability to naturally protect themselves from ROS (Aitken & Fisher, 1994; Hagedorn et al., 2012). In the present work, we studied the effect of several oxidative stress degrees generated by H_2O_2 both on sperm motility and SDF in zebrafish. We found that sperm total motility decreased as H_2O_2 increased, and these results were in agreement with other studies of mammals as bovine (*Bos taurus*) (Bilodeau, Blanchette, Cormier, & Sirard, 2002) and human (Du Plessis et al., 2010) and other fish species as rainbow trout: (Dietrich et al., 2005). In zebrafish, the xanthine-xanthine oxidase system produced a decrease in the total motility regardless of the concentration that was used (Hagedorn et al., 2012). The authors suggested that the

TABLE 2 Effect of different H_2O_2 concentrations on DNA fragmentation and the size of halo after sperm chromatin dispersion test

Group[a]	Fragmented sperm %	Halo size
C	25.0 ± 2.7[a]	4,548 ± 135[a]
L	31.6 ± 2.9[a]	3,483 ± 133[b]
H	48.1 ± 3.0[b]	4,854 ± 127[a]

[a]Control (C), Low (L) and High (H). The C group was incubated for 20 min at 4°C without H_2O_2. The L and H group samples were incubated in 50 and 200 μM H_2O_2 per ml, respectively, for 20 min at 4°C. Different superscripts (a–b) within the same column indicate differences from pairwise comparisons ($p < 0.05$).

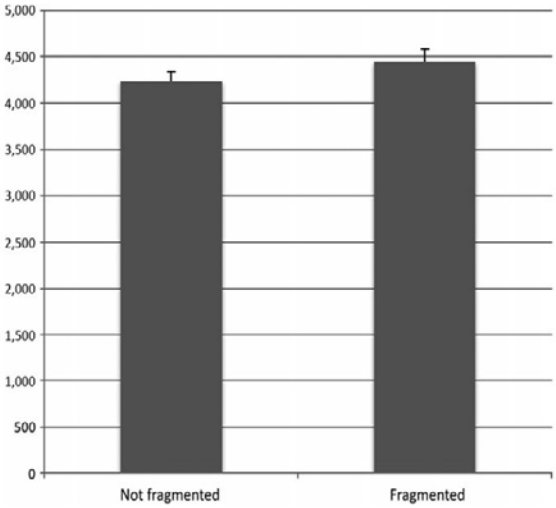

FIGURE 5 Area of halos (pixels) after sperm chromatin dispersion test in spermatozoa categorized as DNA fragmented or not

production of H_2O_2 may be the main ROS in zebrafish (Hagedorn et al., 2012). However, the influence of H_2O_2 on zebrafish sperm motility was not studied. In the common carp (*Cyprinus carpio*) spermatozoa, Gazo et al. (2015) found similar results, but in this case, the motility rate and velocity decreased according to the time and concentration of applied treatment. This detrimental effect of H_2O_2 was evident when we observed the effect of time on sperm motility. Motility of sperm treated with H_2O_2 decreased more rapidly than the control group. No similar information was found in regard to zebrafish, but this result agrees with other studies of carp (Gazo et al., 2015). Hydrogen peroxide not only affected the total motility of sperm, but it also reduced kinetic variables related to velocity (VCL, VSL) and linearity in rainbow trout sperm (Dietrich et al., 2005).

In spite of the high sensitivity to oxidative stress, spermatozoa are proficient generators of ROS (Aitken & Curry, 2011). The high cellular metabolism of sperm required for ATP to obtain flagella movement generates free radicals (Blount, Moøller, & Houston, 2001). In the recent past, Chauvigné, Boj, Finn, and Cerdà (2015) observed in gilthead seabream (*Sparus aurata*) that AQP8b aquaporin acted as a peroxidina to allow the exit of H_2O_2 stored during both osmotic stress and ATP production in sperm motility. In this way, if the action of AQP8b is blocked, H_2O_2 concentrations increase in mitochondria, ATP levels reduce, and finally flagella movement stops (Chauvigné et al., 2015). The same could occur when H_2O_2 concentrations in the medium were artificially increased (L and H groups). Chauvigné et al. (2015) also observed that the supplementation of catalase reverted this deleterious effect of H_2O_2.

The packaging of the sperm DNA renders them resistant to high concentrations of H_2O_2 in comparison with other somatic cells (Lewis & Aitken, 2005). However, despite this packaging, nuclear and mitochondrial DNA are substrates that are sensitive to ROS. Reinardy et al. (2013) observed in zebrafish that SDF increased as H_2O_2 concentrations (10–200 μM) increased. In addition, H_2O_2 incubation (500 mM) significantly increased SDF in rainbow trout sperm (Pérez-Cerezales, Martínez-Páramo, Beirão, & Herráez, 2010). This H_2O_2 concentration is much higher than that used in the present experiment for zebrafish. Dietrich et al. (2005) unexpectedly did not found a dramatic increase in rainbow trout sperm DNA fragmentation after incubation in 1–20 mM H_2O_2 solution. It seems that zebrafish sperm is much more sensitive to H_2O_2 than other species such as rainbow trout. In several mammal species, Villani et al. (2010) observed that H_2O_2 also induced DNA damage in a concentration-dependent manner, and this damage was predominantly single-strand DNA breaks. In the present study, in agreement with previous studies in different species, we observed a progressive increase in SDF as H_2O_2 concentrations increased.

In the recent past, a systematic review of humans indicated that predictive value of SDF tests is not evident (Cissen et al., 2016). It is possible that a more objective evaluation, such as a quantitative evaluation of SDF, could reduce the variability of results. In this way, the p53 ELISA method (Raimondo et al., 2014) and halo area (Sebastian & Raghavan, 2015) have been used as alternative procedures to measure SDF. Several works that used SCDT to determine SDF have classified four levels of DNA damage according to halo size (absence, small, medium, and large), but not halo area [Human: (Absalan & Ghannadi, 2012; Tandara et al., 2014); Pig: (Alkmin et al., 2013); Llama: (Carretero, Lombardo, Arraztoa, Giuliano, & Gambarotta, 2012)]. In the recent past, Sadeghi et al. (2016) evaluated halo areas with two SCD techniques (Halosperm® vs. SDFA) in human sperm using the DNA fragmentation module of the ISAS®. Sebastian and Raghavan (2015) found significant differences in the halo area in spermatozoa treated with endosulfan. No information about this methodology was found in fish. In the present study, we have measured the area of halos after SCDT in zebrafish sperm using ImageJ software, and we did not find major damage to the DNA of the treated groups in comparison with the control group. When we analysed the area of halos of fragmented or un-fragmented sperm, we did not find significant differences. It is evident that the human eye was able to better discriminate between these groups, and additional effort is required for the automatic determination of the SDF using the SCDT.

In conclusion, we found that motility of zebrafish sperm, mainly progressive motility, decreased as H_2O_2 increased, and this deleterious effect was more marked when we studied the effect of time on sperm motility. Moreover, SDF increased as the H_2O_2 concentration increased. However, measurements of the halo area did not agree with the subjective SDF rate.

CONFLICT OF INTEREST

None of the authors have any conflict of interest to declare.

AUTHOR CONTRIBUTION

SS participated in data acquisition, and analysed the data. JP participated in data acquisition and designed the study. JY participated in designing the study, analysed the data and drafted the manuscript. JN participated in data acquisition and drafted the manuscript. CS participated in designing the study and drafted the manuscript. MAS participated in designing the study, analysed the data and drafted the manuscript.

ORCID

Jesús L. Yaniz http://orcid.org/0000-0001-5316-1703
Carles Soler http://orcid.org/0000-0001-9809-2596
Miguel Angel Silvestre http://orcid.org/0000-0001-9260-5792

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Experiment 3

Morphometric comparison by the ISAS® CASA-DNAf systemoftwo techniquesfortheevaluationofDNA fragmentation in human spermatozoa

Sara Sadeghi, Almudena Garcia Molina, Ferran Celma, Anthonio Valverde, Sogol Freidounfar, Carles Soler

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INVITED ORIGINAL ARTICLE

Male Fertility

Morphometric comparison by the ISAS® CASA-DNAf system of two techniques for the evaluation of DNA fragmentation in human spermatozoa

Sara Sadeghi[1], Almudena García-Molina[2], Ferran Celma[1], Anthony Valverde[1,3], Sogol Fereidounfar[2,4], Carles Soler[1,2]

DNA fragmentation has been shown to be one of the causes of male infertility, particularly related to repeated abortions, and different methods have been developed to analyze it. In the present study, two commercial kits based on the SCD technique (Halosperm® and SDFA) were evaluated by the use of the DNA fragmentation module of the ISAS® v1 CASA system. Seven semen samples from volunteers were analyzed. To compare the results between techniques, the Kruskal–Wallis test was used. Data were used for calculation of Principal Components (two PCs were obtained), and subsequent subpopulations were identified using the Halo, Halo/Core Ratio, and PC data. Results from both kits were significantly different ($P < 0.001$). In each case, four subpopulations were obtained, independently of the classification method used. The distribution of subpopulations differed depending on the kit used. From the PC data, a discriminant analysis matrix was obtained and a good *a posteriori* classification was obtained (97.1% for Halosperm and 96.6% for SDFA). The present results are the first approach on morphometric evaluation of DNA fragmentation from the SCD technique. This approach could be used for the future definition of a classification matrix surpassing the current subjective evaluation of this important sperm factor.

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Keywords: DNA fragmentation; Halosperm®; morphometry; principal component analysis; SDFA; subpopulation

INTRODUCTION

In humans, approximately 15% of patients with male factor infertility have normal semen analysis results, and so a definitive diagnosis of male infertility often cannot be made solely from results of routine semen analysis.[1] This implies that new seminal parameters must be included in the routine analysis for discriminating other causes of male infertility.

The possible significance of DNA fragmentation on fertility was indicated some years ago.[2–5] To evaluate this semen trait, different techniques have been developed,[6,7] including the TUNEL (Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP Nick End Labeling) assay,[8–10] the Comet assay,[10–12] the chromomycin A3 test,[13,14] Acridine Orange metachromatic staining,[15–17] DNA Breakage Detection-Fluorescence *In Situ* Hybridization,[18] the SCSA (Sperm Chromatin Structure Assay) test,[19] and the SCD (Sperm Chromatin Decondensation) test.[20]

DNA fragmentation in human sperm samples after evaluation by the Comet technique is higher in infertile males than fertile males, and spermatozoa with abnormal morphology and low levels of motility have more DNA damage than normal cells.[5] By using the TUNEL technique, it has been demonstrated that specific abnormal sperm morphology can be correlated with chromosomal abnormalities and the level of DNA fragmentation in human spermatozoa.[21] Development of simple kits for the diagnosis of DNA fragmentation has increased the number of studies on the significance of DNA fragmentation in several species,[22] but there is some controversy over the diagnostic significance of the differential tests, making it difficult to decide which is the best to use.[23,24]

Two commercial kids have been developed around the SCD technique: Halosperm® (Halotech, Madrid, Spain) and SDFA (ACECR, Tehran, Iran). The purpose of the present study was to compare the results from these commercial kits, by performing a morphometric analysis with the ISAS® v1 DNA fragmentation module (Proiser, Valencia, Spain). These morphometric data were used, for the first time to our knowledge, to define mathematical clusters that provide a classification matrix of different subpopulations of sperm head DNA-reacted cells.

MATERIALS AND METHODS

Study population

Seven volunteers signed informed consent form to participate and have their semen used in the study. Semen samples were collected by masturbation after sexual abstinence for 3–5 days. Each sample was collected in a clean 60-ml wide-mouthed universal container and stored at 37°C in an incubator for 30 min to allow liquefaction.

[1]Department of Functional Biology and Physical Anthropology, University of Valencia, 46100 Burjassot, Valencia, Spain; [2]R+D Department, Proiser R+D, Scientific Park, Universitat de València, 46980 Paterna, Valencia, Spain; [3]Technological Institute of Costa Rica, San Carlos Campus, School of Agronomy, 223-21001 Alajuela, Costa Rica; [4]Department of Botany, Kharazmi University, Tehran, Iran.
Correspondence: Dr. C Soler (carles.soler@proiser.com)

Assessment of DNA fragmentation

Two commercial kits were used to assess the level of sperm head DNA fragmentation by the SCD approach: the Halosperm® test (Halotech DNA, S.L., Madrid, Spain) and the Sperm DNA Fragmentation SDFA test (ACECR, Tehran, Iran). For both tests, semen samples were diluted with Sydney IVF Sperm Medium (Cook® Medical, Bloomington, IN, USA) to a sperm concentration of 5–10 × 10^6 cells ml^{-1}. Agarose gel from the kit (500 µl for Halosperm or 100 µl for SDFA) was incubated in an Eppendorf tube for 5 min at 90–100°C to melt the agarose and then 5 min at 37°C in temperature-controlled water bath after which 25 µl (Halosperm test) or 50 µl (SDFA test) of the semen sample was added into an Eppendorf tube and mixed carefully. For both tests, 15 µl of the mixture was placed onto a kit-provided super-coated slide, placed on a cold surface, and covered with a 22 mm × 22 mm coverslip. Slides were kept for 5 min at 4°C in a refrigerator to create a microgel with the contained spermatozoa.

For the Halosperm test, coverslips were then carefully removed, and the slides immersed into acid denaturation solution for 7 min, transferred to a tray of the kit's lysing solution for 25 min incubation, rinsed with distilled water and dehydrated for 2 min in each of 70%, 90%, and 100% (v/v) ethanol. After being dried, the slides were stained with Diff-Quik (Medion Diagnostics, Düdingen, Switzerland) in a horizontal position, first in Eosin (red color) for 7 min, then in Azur B (blue color) for 7 min, and finally rinsed in distilled water and allowed to dry at room temperature.

For the SDFA test, coverslips were carefully removed, and a few drops of solution A were added to the slide, which was incubated for 7 min. Slides were transferred to solution B and incubated for 15 min, rinsed with distilled water and dehydrated for 2 min in increasing concentrations of ethanol (70%, 90%, and 100%). After being air-dried, the slides in a horizontal position were stained sequentially with the kit's staining solutions: solution C for 75 s, solution D for 3 min, and solution E for 2 min, then rinsed in distilled water and allowed to dry at room temperature.

Morphometric analysis

Analyses were conducted by using the DNA fragmentation module of the ISAS® v1 (Proiser R+D S.L., Paterna, Valencia, Spain) CASA-DNAf system. The camera used was Proiser 782 m (Proiser R+D S.L.) attached to a microscope UB203 (UOP/Proiser, Paterna, Valencia, Spain). Images were captured through a 40 × bright field objective (AN 0.7) with resolution of the analyzed images of 0.21 µm/pixel for both axes. The software renders three morphometric parameters: the total Halo and central Core areas (μm^2), distinguished by the intensity of staining (**Figure 1**), and the Ratio between them.

Statistical analysis

Clustering procedures were performed on the datasets to identify sperm subpopulations from the Halo parameter values and the Ratio criteria. In both cases, the parameter values were examined using a nonhierarchical clustering procedure (k-means model and Euclidean distance), to classify the spermatozoa of the dataset.[25] The first step was to perform a principal component analysis (PCA) of the DNA fragmentation data. The morphometric database comprised a total number of 1775 spermatozoa. To select the number of principal components that should be used in the next step of analysis, the criterion of selecting only those components with an eigenvalue (variance extracted for that particular principal component) >1 (Kaiser criterion) was chosen. The second step was to perform a two-step cluster procedure with the sperm-derived indices obtained after the PCA to determine the subpopulation structure.

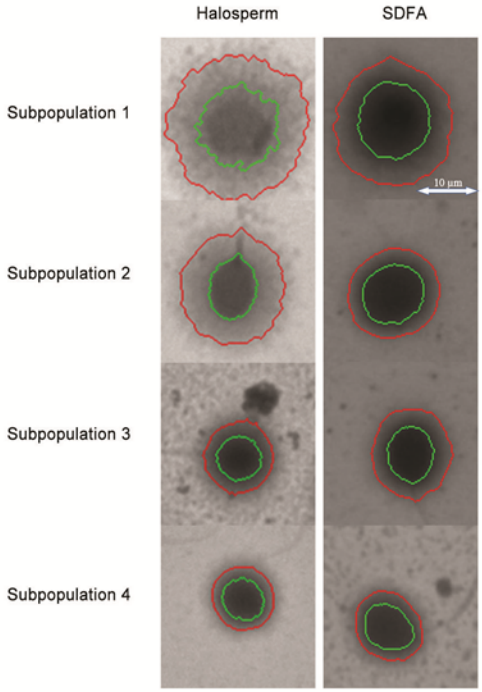

Figure 1: Images of DNA-reacted cells after treatment with both kits (Left column Halosperm, Right column SFDA). One representative cell from each subpopulation is shown (Top-to-bottom – SP1 large, SP2 large-medium, SP3 medium-small, SP4 small). Scale bar = 10 µm applicable to each figure.

The relative distribution frequency of spermatozoa belonging to each subpopulation for each patient was analyzed by the Chi-squared and Mantel–Haenszel Chi-squared tests. The morphometric data on the DNA fragmentation and the multivariate method were first tested for normality and homoscedasticity by using Shapiro–Wilks and Kolmogorov–Smirnov tests, respectively. Because no parameters satisfied either criterion, nonparametric analyses were performed with the Kruskal–Wallis test. The results were presented as mean ± standard deviation (s.d.). Statistical significance was considered at $P < 0.05$.

Discriminant analysis[26] was performed from the principal components and the classification by subpopulations of the DNA fragmentation to obtain a classification matrix. The analysis was done considering the morphometric data independently, with a linear stepwise procedure to identify those parameters that were most useful for classifying individual cells into one of the four subpopulations. In all cases, principal component vectors were added to the discriminant function variables to obtain significantly better discrimination. It was found in all cases that all the variables were useful for discrimination. The classification matrix obtained after this discriminant analysis was applied to the whole population to establish the proportion of cases in each category by each DNA fragmentation kit.

All data were analyzed using InfoStat Software v2008 (University of Córdoba, Córdoba, Argentina) for Windows.[27]

RESULTS

Correlation of morphometric parameter values between kits

Comparison of the total number of cells analyzed from both kits produced a significant correlation between considered morphometric data ($P < 0.001$) although the correlation values between both kits were not enough to

permit to extrapolation of one set of data from the other. The value of *r* for Halo was of 0.54, for Core of 0.44, and for the Halo/Core Ratio of −0.35.

Principal component analysis

For the two kits considered, principal component analysis of the three parameters analyzed (Halo, Core, and Halo/Core Ratio) rendered two PCs. In the case of Halotech, PC1 was related to the three parameters and explained 80% of the variance. PC2 was essentially related to Ratio, explaining the remaining 20% (**Table 1**). In the case of SDFA, PC1 was positively related to the Halo and Core areas, and negatively to the Ratio, explaining 79% of the variance, and PC2 was related to Ratio and Halo, explaining 20% (**Table 1**).

Subpopulation structure

The distribution of DNA-reacted cells in the subpopulations was made on the basis of the Halo area, the Halo/Core Ratio, and from the PC data. Independently of the classification criteria and the kit used, four subpopulations were found. In all cases, the subpopulations comprised four size classes: SP1, large; SP2, large-medium; SP3, medium-small; and SP4, small (**Table 2** and **Figure 1**). Only the values of Ratio showed no differences between SP1 and SP2 from Halosperm samples with the Halo classification method. No differences in Core values were found between SP2, SP3, and SP4 for the SDFA samples for Core values examined by the Ratio classification method or between SP3 and SP4 for Ratio with the PC classification method (**Table 2**).

Table 1: Principal component analysis of morphometric data from DNA-reacted cells for each DNA fragmentation kit application

Parameter	*Halosperm*		*SDFA*	
	PC1	*PC2*	*PC1*	*PC2*
Halo	0.6		0.6	0.6
Core	0.6	−0.5	0.6	
Ratio	0.5	0.8	−0.5	0.8
Explained variation (%)	80	20	79	20

Only eigenvalues >0.3 are shown. PC: principal component; SDFA: sperm DNA fragmentation assay

For both kits and methods, SP1 was the less frequent with the exception of the SDFA kit for the Ratio method. The distribution of the other SPs depended on the kit and method (**Table 2**). With the Halo area as the classification criterion, the larger reacted cells (SP1) with both kits comprised similar and low percentage of cells, but SP2 was <10% for Halosperm and >20% for SDFA. For the Halosperm kit, SP4 was more abundant than SP3, but for SDFA, SP3 was more frequent than SP4 (**Table 2**). The distribution of subpopulations in patients varied both between classification techniques and kits used (**Table 3**).

Discriminant analysis

For this study, we only used the data from PCA. During the previous subpopulation analysis, each cell was assigned to one of the four subpopulations, and this assignment was used to define the canonical cells for the discriminant analysis performance. Fisher discriminant linear coefficients for both kits were obtained (**Table 4**). After reclassification of canonical cells following the Fisher matrix, the percentage of well-classified cells was 97.2 for Halosperm and 96.9 for SDFA. In the Halosperm samples, all the cells of SP1 were well classified while 5.2% of the cells from SP4 were classified as belonging to SP3. In the SDFA samples, both SP1 and SP2 showed 100% correct classification while 9.7% of the SP3 were classified as SP2 (**Table 5**).

DISCUSSION

Different studies have shown that DNA fragmentation evaluated by the SCD technique is a good parameter for predicting fertility in humans,[28–30] even better than "standard parameters" when combined with mitochondrial membrane potential.[31] In addition to studies on fertility, this technique has been applied to different clinical and toxicological situations such as varicocele,[32] ejaculatory abstinence,[33] cigarette smoking and alcohol consumption,[34] and genitourinary infection.[35] From SCD criteria, independently of the test kit used, sperm cells with very small halos or without halos, as well as degraded sperm cells, are classified as containing fragmented DNA, and cells with intermediate or large halos are not considered fragmented.[20,22,29,36] In some papers, the description is somewhat more accurate, including

Table 2: Morphometric values (mean±s.d.) by subpopulation for each parameter presented by the kit used and the classification method

CM/P	*Halosperm*				*SDFA*			
	%	*Halo*	*Core*	*Ratio*	*%*	*Halo*	*Core*	*Ratio*
Halo (μm²)								
SP1	2.9	378.2±55.7^{a,m,x}	125.7±29.1^{a,m,x}	3.1±0.5^{a,m,x}	6.3	318.2±54.4^{a,n,x}	299.0±64.8^{a,n,x}	1.1±0.3^{a,n,x}
SP2	7.8	228.7±33.6^{b,m,x}	75.5±13.9^{b,m,x}	3.1±0.5^{a,m,x}	23.7	199.8±25.7^{b,n,x}	158.9±51.2^{b,n,x}	1.4±0.6^{b,n,x}
SP3	31.2	117.7±20.9^{c,m,x}	50.7±9.4^{c,m,x}	2.3±0.3^{b,m,x}	36.4	126.5±19.2^{c,n,x}	85.4±33.4^{c,n,x}	1.7±0.5^{c,n,x}
SP4	58.1	65.6±16.8^{d,m,x}	31.8±7.6^{d,m,x}	2.1±0.3^{c,m,x}	33.6	58.0±19.6^{d,n,x}	31.8±14.9^{d,n,x}	1.9±0.4^{d,n,x}
Ratio								
SP1	5.8	244.6±88.4^{a,m,y}	70.2±26.3^{a,m,y}	3.5±0.3^{a,m,y}	38.4	188.4±73.3^{a,n,y}	178.6±71.3^{a,n,y}	1.1±0.03^{a,n,x}
SP2	18.4	150.2±91.3^{b,m,y}	55.9±31.8^{b,m,y}	2.6±0.2^{b,m,y}	9.1	125.0±70.3^{b,n,y}	46.9±27.0^{b,n,y}	2.7±0.4^{b,m,y}
SP3	46.8	92.6±35.7^{c,m,y}	41.0±15.3^{c,m,y}	2.2±0.1^{c,m,y}	23.1	103.0±56.2^{c,m,y}	48.0±26.0^{b,n,y}	2.1±0.1^{c,m,y}
SP4	29.0	64.1±23.3^{d,m,y}	35.5±12.7^{d,m,y}	1.8±0.1^{d,m,y}	29.4	86.7±40.8^{d,n,y}	49.0±21.9^{b,n,y}	1.8±0.1^{d,m,x}
PC								
SP1	3.2	343.8±88.3^{a,m,x}	126.4±25.5^{a,m,x}	2.7±0.4^{a,m,z}	8.7	297.3±58.1^{a,n,x}	284.3±56.8^{a,n,x}	2.4±0.4^{a,n,y}
SP2	7.3	223.5±71.2^{b,m,x}	66.4±20.1^{b,m,x}	3.4±0.4^{b,m,z}	29.5	157.3±38.4^{b,n,z}	148.6±37.3^{b,n,z}	1.9±0.2^{b,n,z}
SP3	49.4	89.6±31.3^{c,m,y}	37.5±11.3^{c,m,z}	2.4±0.2^{c,m,x}	18.9	148.6±57.2^{b,n,z}	64.2±27.5^{c,n,z}	1.1±0.01^{c,n,z}
SP4	40.1	79.8±33.7^{d,m,z}	40.8±14.0^{d,m,z}	1.9±0.2^{d,m,z}	42.9	76.3±33.0^{c,n,z}	41.4±18.9^{d,n,z}	1.1±0.01^{c,n,y}

$^{a-d}$Different superscripts indicate significant differences between subpopulations within each CM for each parameter and kit; $^{m-n}$Different superscripts indicate significant differences between kits for each subpopulation within each CM and parameter; $^{x-z}$Different superscripts indicate significant differences between CM for each subpopulation for each parameter and kit. Significant differences were considered at $P<0.05$ after Kruskal–Wallis test. CM: classification method; P: parameter; PC: principal component; Ratio: halo/Core; SP: subpopulation; %: percentage of total sperm number comprising each SP; s.d.: standard deviation; SDFA: sperm DNA fragmentation assay

Table 3: Distribution of subpopulations (%) for each volunteer in each kit from different classification methods

	Halosperm				*SDFA*			
	SP1	*SP2*	*SP3*	*SP4*	*SP1*	*SP2*	*SP3*	*SP4*
Halo CM								
1*,x	0.0	0.0	34.4	65.6	1.1	38.6	50.0	10.2
2x	24.8	54.5	13.9	6.9	31.5	40.7	17.6	10.2
3*,x	0.0	0.0	0.0	100.0	0.0	0.0	52.1	47.9
4	0.0	3.5	29.9	66.7	0.5	9.2	32.4	57.8
5*,x	0.0	0.6	57.1	42.2	3.2	33.8	40.9	22.1
6*,x	0.7	5.6	47.6	46.2	10.4	18.7	29.9	41.0
7*,x	0.0	0.0	10.7	89.3	0.0	22.0	43.3	34.7
Ratio CM								
1*,y	0.0	33.3	61.5	5.2	54.5	0.0	4.5	40.9
2*,y	49.5	34.7	9.9	5.9	53.7	12.0	21.3	13.0
3*,y	1.8	5.3	77.2	15.8	4.2	0.0	37.5	58.3
4*	0.0	4.0	40.8	55.2	8.6	14.6	33.0	43.8
5*,y	0.0	20.5	47.8	31.7	59.7	4.5	12.3	23.4
6*,y	0.7	24.5	36.4	38.5	42.5	10.4	21.6	25.4
7*,y	0.7	14.1	67.8	17.4	40.0	12.0	30.7	17.3
PC CM								
1*,z	0.0	1.0	86.5	12.5	6.8	47.7	5.7	39.8
2*,z	22.8	55.4	8.9	12.9	39.8	13.9	32.4	13.9
3*,z	0.0	1.8	84.2	14.0	0.0	4.2	10.4	85.4
4*	1.0	0.0	26.9	72.1	1.1	7.6	27.0	64.3
5*,z	0.6	0.0	52.8	46.6	5.2	53.2	11.7	29.9
6*,z	2.1	4.9	42.0	51.0	10.4	32.1	17.9	39.6
7*,z	0.0	0.7	73.8	25.5	1.3	38.7	18.0	42.0

*For each volunteer significant differences in subpopulation distribution between kits for each CM, x-zFor each volunteer significant differences in subpopulation distribution between CM for each kit. Significant differences by Chi-squared test were considered at $P<0.05$. CM: classification method; PC: principal component; Ratio: halo/Core; SP: subpopulation; SDFA: sperm DNA fragmentation assay

Table 4: Discriminant linear coefficients classification matrix (Fisher) from principal component data

	SP1	*SP2*	*SP3*	*SP4*
Halosperm				
PC1	4.9546562	2.9556255	−0.1947948	−0.6903666
PC2	−1.7570925	1.2368074	0.3601852	−0.5285625
SDFA				
PC1	3.3050860	1.1265700	−0.8204570	−1.0799150
PC2	0.6298914	−0.6116444	1.1426632	−0.2098324

SP: subpopulation; SDFA: sperm DNA fragmentation assay; PC: principal component

Table 5: Percentage of cells of the reference population assigned to each class after discriminant analysis of the principal component data

	SP1	*SP2*	*SP3*	*SP4*	*Total*
Halosperm					
SP1	100	0.0	0.0	0.0	100
SP2	0.0	97.0	3.0	0.0	100
SP3	0.0	0.2	96.7	3.1	100
SP4	0.0	0.0	5.2	94.8	100
SDFA					
SP1	100	0.0	0.0	0.0	100
SP2	0.0	100.0	0.0	0.0	100
SP3	0.0	9.7	90.3	0.0	100
SP4	0.0	0.0	4.1	95.9	100

Overall 97.1% and 96.6% of the reference sperm population by Halosperm and SDFA, respectively, were classified correctly. SDFA: sperm DNA fragmentation assay; SP: subpopulation

references to relatives sizes, but proper morphometric measures have not been done.[37]

As with other seminal parameters, when subjective evaluation is done, relative criteria are used. How is a small halo to be defined? Just because it is not medium or big, but how is it defined? The actual expanse of the Halo is a continuous variable that cannot be reduced to one discrete value just by choice. In the present work, it was demonstrated that with appropriate statistics, the classification can be based on the real nature of the variables. The four subpopulations we have found in all cases paralleled the subjective evaluations of "no halo," "small halo," "medium," and "large halo," respectively, but on the basis of mathematical data, they can be applied to future classifications. Correlation studies between both approaches must be done. The differences observed in classification criteria between kits used indicate that even with results from the same principle (SCD), differences could originate from the composition of the solutions. For future work, it is thus necessary to mention specifically the technique used when results are presented.

Instead of using just one of the morphometric parameters offered by CASA-DNAf systems for classification, it is better to integrate them through the use of principal component analysis, and for this reason, we have used this approach for the definition of the classification matrix that can be used for the future work. We acknowledge that the number of cases and spermatozoa presented here is insufficient to establish a definitive classification matrix, but this paper represents a start in the rigorous objective sorting of spermatozoa processed to provide assessment of their nuclear DNA fragmentation. More work will be done in the way described here.

CONCLUSION

The present data are the first demonstration of a new evaluation of DNA fragmentation from morphometric criteria, but we have not aimed to compare the former definition of fragmentation with the new purpose. Future work on more data following this method will provide a classification matrix to be used in future evaluation, by the use of automatic CASA-DNAf systems, of the impact of DNA fragmentation on male fertility in both clinical and research work.

AUTHOR CONTRIBUTIONS

SS and CS conceived and designed the experiments; AGM, FC, and SF performed the experiments; AV and CS analyzed the data; CS wrote the paper.

COMPETING INTERESTS

CS is Professor at Valencia University and acts as Scientific Director of Proiser R+D S.L Research and Development Laboratory. Neither he nor the other authors have interests that influenced the results presented in this paper.

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FINAL COMMENTS

We consider necessary to review the history of the present Thesis development. The main objective was from the beginning the study of ROS on semen quality using the zebrafish as an experimental model. Nevertheless, before starting the experiments about ROS influence on spermatozoa of zebrafish, it was needed to establish the conditions of sperm activation and conservation in zebrafish. The revision of the literature indicated that few works contributing to the evaluation of motility and its duration over time in zebrafish were available, and no one on sperm kinematics and DNA fragmentation. As a consequence, we decided to begin our experiments analysing the zebrafish sperm motility and kinematics under the experimental conditions. In fact, sperm motility and its duration is one of the most important factors in fish fertility, which clearly is related to osmolality of activation medium. All these aspects were the nucleus of the first paper which included in the present Thesis.

The main results of this first paper showed that the most sensitive factor in sperm motility is the initial velocity parameters and their duration over the activation time. Sperm motility rate was higher and its duration was prolonged in time with high osmolality ranges of activation medium, also all the velocity dependent parameters show relevant affect by activation media in high osmotic concentration. Furthermore, higher sperm motility rates were observed for the fresh semen samples, and the drop of total sperm motility over time seemed more pronounced in the sperm samples stored at 4ºC. Moreover, we observed spermatozoa activated with a high osmolality medium showed higher values in motility and kinetic parameters in comparison with a low osmolality medium.

Once the optimal zebrafish sperm activation protocol was defined, we studied the oxidative stress effects of H_2O_2 at different levels on spermatozoa of zebrafish, considering this species as an experimental model. This work constituted the second paper of the Thesis and conduct to demonstrate that zebrafish sperm motility, particularly progressive motility, decreased as H_2O_2 increased, and this deleterious effect was more marked when we studied the effect of time on sperm motility. In addition, also the levels of DNA fragmentation, measured subjectively accordingly with the detection kit used, were increased as the H_2O_2

concentrations increased. However, quantitative measurements of the halo area did not agree with the subjective SDF rate, as it was observed in other species.

In parallel to the work developed on zebrafish model, our group was working also in other species, including human. The availability of a new kit for DNA fragmentation in humans offered the possibility to analyse the differences between this kit and that used in the zebrafish experiments. The results showed that both kits are measuring the SDF level, even they presented differences in the morphometric characteristics of the reacted cells. The differences observed in classification criteria between kits used indicate that even with the results from the same principle (SCD), differences could originate from the composition of the solutions. In addition, a new approach for the classification of SDF was used, following cluster analysis (in two steps), which revealed that the actual expanse of halo is a continuous variable that cannot be reduced to one discrete value by choice. It was demonstrated that, with appropriate statistics, the classification can be based on the real nature of the variables. The four subpopulations we found in all cases patrolled the subjective evaluation of “no halo”, “small halo”, “medium halo” and “large halo” respectively, but on the basis of mathematical data. This new classification was proposed as alternative to the classical subjective one and will be very useful in the future human sperm SDF evaluation. Our idea was to apply the same protocol to our experimental model, but, in this time, the maintenance of the animals in the lab was no more available.

Finally, even the results cannot be included as a part of the present Thesis, we have changed the animal model from zebra fish to goat. In last experiment using the Murciano-Granadina male goats to evaluate the correlation between semen cooled conservation conditions and DNA integrity, oxidation level, and mitochondrial activity in sperm using flow cytometry technologies

CONCLUSIONS

- Spermatozoa activated with high osmolality medium displayed higher values in motility and kinetic parameters.

- With respect to individual or pool samples, no relevant changes were observed in total motility or in kinetic parameters.

- Total motility and velocity of sperm showed significant lower values after cool storage.

- Motility of zebrafish sperm, particularly progressive motility, decreased as H_2O_2 increased, and this deleterious effect was more marked with longer incubation times.

- The subjective SDF levels increased as the H_2O_2 concentrations increased in medium, but, the measurements of the halo area did not agree with the subjective SDF rate.

- Different kits based on SCD principle rend different results, being all of them representative of SDF in human samples.

- Four subpopulations based on morphometric data obtained from samples prepared with Halosperm and SDFA defined SDF level and can be used in replacement of former subjective evaluation.

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Print name: Carles Soler

Date: 11/09/2018

Address:

University of Valencia, avenida DR.Moliner,50
46100 Burjassot, VALENCIA

Send signed form to: Asian Journal of Andrology Editorial Office, Shanghai Institute of Materia Medica, Room 302, Building 16, 294 Tai-Yuan Rd, Shanghai 200031, China.
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ANNEX 2: Experiment 4

Effect of sperm concentration and storage temperature on motility and DNA fragmentation, mitochondrial activity, oxidation level and membrane integrity of cooled buck spermatozoa

The reduction of sperm concentration in refrigerated insemination doses, as well as its combination with different storage temperatures, has recently been studied in cattle (Murphy et al., 2013, 2018). In goats, the use of cooled insemination doses is relatively common; however, no studies with the aim of improving the conditions of liquid storage of goat semen were found in the literature.

We studied the effect of storage temperature, and sperm concentration on the in vitro semen quality. For this, three experiments were performed. Briefly, adult Murciano-Granadina male goats were reared at Centro de Tecnología Animal, Instituto Valenciano de Investigaciones Agrarias (Segorbe, Spain). Semen was collected by means of a pre-warmed artificial vagina.

Study 1: Effect of different concentrations and temperature during liquid storage on sperm motility

Sperm preservation was studied under different conditions. Each individual male ejaculate was diluted in supplemented skimmed milk to different concentrations (250×10^6 sperm/mL **[1/2]**, 166.7×10^6 sperm/mL **[1/3]** and 50×10^6 sperm/mL **[1/10]**) and stored at different temperatures (5ºC and 17ºC). Sperm were evaluated at three different incubation times (0, 24 and 48 hours).

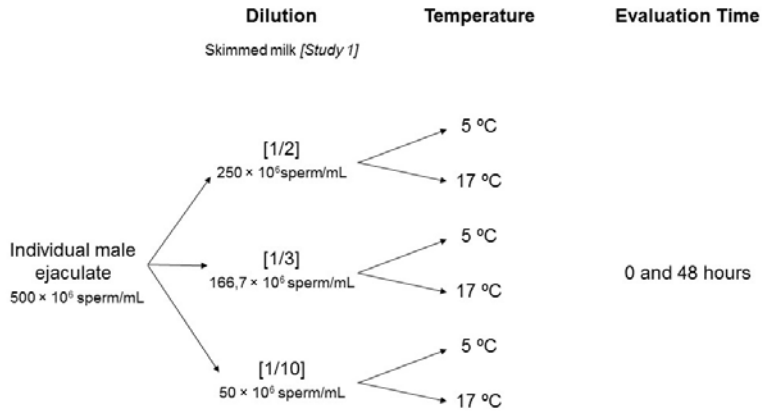

Figure 1: Effect of concentration, and temperature during liquid storage.

Study 2: Effect of different concentration, centrifugation and temperature on sperm motility and DNA fragmentation

Half of the three-ejaculate pool was washed, by centrifugation, in order to study whether the removal of seminal plasma improved the preservation of sperm. As previously described, sperm samples were further diluted in skimmed milk to different concentrations (250×10^6 sperm/mL **[1/2]** and 50×10^6 sperm/mL **[1/10]**). Each sperm sample, control and study was conserved at two different temperatures (5ºC and 17ºC) in darkness. Sperm were evaluated at two different times (0 and 48 hours).

After the motility evaluation at 48 hours, all the samples were centrifuged to wash the skimmed milk and suspended in phosphate-buffered saline (PBS) and frozen at -80ºC until all the three replicates were prepared for the DNA fragmentation procedure.

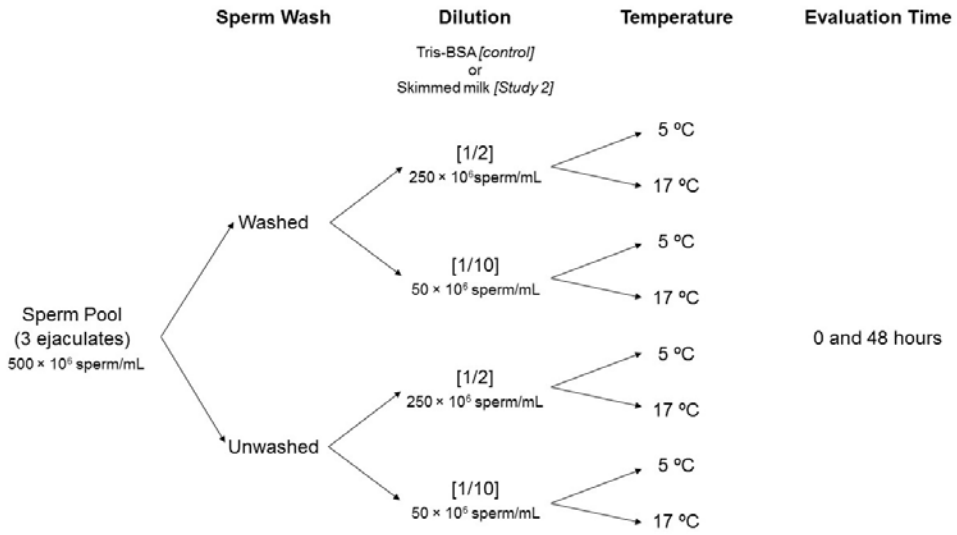

Figure 2: Effect of concentration, centrifugation and temperature on sperm

Study 3: Effect of different sperm concentration and time of storage at 4ºC on motility

We used a pool of ejaculates from three males. Sperm samples were further diluted in skimmed milk to different concentrations (250 × 10^6 sperm/mL **[1/2]** and 50 × 10^6 sperm/mL **[1/10]**). Motility, mitochondrial status, oxidation level and membrane integrity of spermatozoa were evaluated at two different times (0 and 48 hours).

Evaluation of sperm samples

Seminal motility variables test

Sperm analysis was performed using the CASA-mot system (ISAS® v1.2; PROISER S.L., Paterna, Spain) and a Basler digital camera (A780-54fm) at a frame rate of 25 images per second.

Oxidation level and integrity of membrane (Sellem et al., 2015)

Reactive oxygen species level was measured by Easykit 3 (Ref. 025157; IMV Technologies). Results were expressed as four groups: oxidised or not and membrane-intact or damaged sperm.

Mitochondrial status (Sellem et al., 2015)

Mitochondrial status was measured by Easykit 2 (Ref. 024864; IMV Technologies). Results were expressed as two groups: sperm with low (depolarised) or high membrane potential.

Flow cytometry

Mitochondrial status, oxidation level and membrane integrity tests were evaluated using a flow cytometer BD LSRFortessa. It contains three lasers: yellow-green 561 nm, yellow 586/15 nm, and green 530/30 nm. A minimum of 3000 cells/replicate and groups were assessed.

DNA fragmentation test

The level of sperm DNA fragmentation was assessed using a commercial variant of the sperm chromatin dispersion test (Halomax®; ChromaCell SL, Madrid, Spain). All the samples were evaluated, stained and visualised. Our experiment was conducted comparing the best condition to preserve goat semen without any freezing process. Each sample was processed in different conditions, evaluating sperm motility and DNA fragmentation in various groups of semen samples.

To check the effect of temperature, incubation time, dilution and seminal plasma, each pool semen sample was divided into eight groups: 5°C and 17°C, 0 h and 48 h, 1/2 and 1/10 of sperm concentration and washed (suspended in skimmed milk as an extender) and non-washed plasma samples. After 48 hours and the motility test, all the groups were centrifuged at 1,000 x g for 10 minutes to wash the skimmed milk and then suspended with PBS to freeze in -80°C.

In brief, an aliquot of a semen sample was diluted to 15–20 million/mL in PBS. Gelled aliquots of low melting point agarose in Eppendorf tubes were provided in the kit, each one to process a semen sample. Eppendorf tubes were placed in a water bath at 90°–100°C for five minutes to fuse the agarose, and then in a water bath at 37°C for five minutes until the temperature equilibrated. Then, aliquot 25 µl of each sperm sample was placed in an empty Eppendorf tube and mixed gently with 50 µl of liquefied agarose. The tubes were maintained at 37°C. A drop of 2 µl of the sperm gel suspension was placed onto the marked wells and covered with a 24 x 24 mm glass coverslip. The slides were placed on a cold plate in the refrigerator (4°C) for five minutes to allow the agarose to produce a microgel with the sperm cells embedded within. The coverslips were gently removed and the slides immediately immersed horizontally in lysis solution, incubated for five minutes and drained. The slide was washed with distilled water and dehydrated by flooding in 70% and 100% ethanol for two minutes in each and allowed to dry before staining.

Staining and visualisation

All the slides were stained just before visualisation with fluoGreen® (halotech) fluorochrome, mixed in a 1:1 proportion of green fluorochrome and mounting medium and 3 µl of the mix used over the slide, which was covered with a coverslip. All slides were visualised and counted using fluorescence microscopy. About 300 sperm per sample were counted.

Results

There was no significant change between washed and unwashed (centrifugation) spermatozoa on DNA fragmentation. There were more DNA fragmentation in sperm samples with minor dilution and concentration 1/2 (4.0 ± 0.29^{a}), 1/10 (2.6 ± 0.2^{b}), $p > 0.05$. However, the lower sperm concentration (1/10) showed a greater rate of spermatozoa with low (depolarised) membrane potential of mitochondria. Murphy et al. (2013) did not find any effect on the mitochondrial activity of sperm. The 1/2 group were no different to the control group. As we expected, the rate of sperm with low mitochondria membrane potential increased as storage time increased. This fact was similar for the parameter "alive oxidised sperm". This parameter measured the capacity of spermatozoa to react to a mild oxidation after H_2O_2 exposure (Barrier-Battut et al., 2016). This parameter was correlated to in vivo fertility in cattle (Sellem et al., 2015; Barrier-Battut et al., 2016). The "alive oxidised sperm" parameter dropped as storage time increased, particularly after 48h. However, the sperm doses with lower sperm concentration showed the highest values of the "alive oxidised sperm" parameter. More studies are required to elucidate the relationship between these parameters and in vivo fertility.

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RESUMEN de la tesis Doctoral

Infertilidad con el factor masculina

Actualmente los investigadores son más conscientes de la importancia del factor masculino en la etiología de la infertilidad humana y ahora reconocemos que la función defectuosa de los espermatozoides es una de las causas más comúnmente definidas. La infertilidad debido al factor masculino es la razón de la infertilidad en el 20-30% de las parejas infértiles, al tiempo que contribuye al 50% de los casos en general. Aproximadamente el 7% de los hombres en todo el mundo son infértiles (Bui et al., 2018; Mosher y Pratt, 1991).

La Organización Mundial de la Salud (OMS) publica periódicamente manuales para el examen de laboratorio y el procesamiento del semen humano. La última actualización, que proporcionó mejoras sustanciales en la forma de evaluar los parámetros seminales, se realizó en 2010. En su última edición, los valores de referencia del análisis del semen son notablemente más bajos que los de las ediciones anteriores. En el estudio de la fertilidad masculina en todas las especies animales, la movilidad espermática, junto a la concentración y morfometría son los parámetros principales y más básicos en la evaluación de la calidad de los espermatozoides. La infertilidad masculina habitualmente se asocia con tasas anormales de estos parámetros seminales. Aun teniendo, los espermatozoides tasas adecuadas de todos estos parámetros no se puede garantizar la capacidad de fecundar y/o desarrollar un cigoto.

El Sistema de análisis automatizado espermático (Computerized Assisted Sperm Analysis; CASA)

Con la introducción de la tecnología de CASA a finales de la década de 1970, el objetivo era vencer estos problemas y las aprobaciones de investigación se obtuvieron tanto en el campo científico como en el clínico. En una tentativa por hacer que la evaluación de la calidad del semen sea más objetiva y detallada, se han desarrollado diferentes herramientas de CASA. Overstreet et al. (1979) describen el sistema CASA como un método simple y objetivo para obtener un conjunto bien definido y diversificado de características del movimiento de espermatozoides (CASA - mot). Usando CASA-mot, se puede obtener varios

parámetros específicos de movilidad que describen los movimientos de espermatozoides en una manera más específica. Además, la clasificación en espermatozoides móviles e inmóviles puede establecer parámetros de velocidad bien definidos. Además de los esfuerzos por optimizar los resultados técnicos de los sistemas CASA, se ha investigado la relevancia biológica de los parámetros de CASA-mot en términos de predicción de la fertilidad masculina (Bompart et al., 2018; Larsen et al., 2000). Los sistemas CASA-mot proporcionan una evaluación más objetiva de la movilidad espermática que la estimación visual, así como información adicional como las variables cinéticas. Sin embargo, numerosos factores no relacionados con las muestras de semen podrían verse influenciados en el análisis de la movilidad de los espermatozoides, como el sistema CASA comercial que usamos y el efecto de la configuración técnica, el número de imágenes, la velocidad de adquisición de las imágenes o el tipo de cámara que se utilizó para el análisis. Las concentraciones espermáticas, los diluyentes utilizados y el volumen de las muestras semen también puede influir de manera notable en los resultados. Por consiguiente, es importante estandarizar las condiciones de medida para la evaluación de las muestras semen y poder comparar valores entre diferentes estudios (Del Gallego et al., 2017). Por otro lado, está claro que el examen rutinario del semen y los espermatozoides en la eyaculación no puede evaluar otros parámetros importantes en el proceso de fecundación como es capacitación espermática o detectar la fragmentación del ADN o la disfunción mitocondrial de los espermatozoides. El ADN del espermatozoide juega un papel crítico en el desarrollo normal del embrión ya que la información genética transmitida a la próxima generación depende de la integridad del ADN del esperma.

Estructura de cromatina espermática en mamíferos y peces

En humanos y la mayoría de los mamíferos, la cromatina de espermatozoides durante la espermatogénesis, experimentan una transformación profunda, en la cual se elimina el 85-95% de las histonas y se substituyen por protaminas. Las protaminas son aproximadamente la mitad del tamaño de las histonas. Los núcleos de células espermáticas simplemente no tienen el volumen necesario para este tipo de empaquetamiento. La sustitución de la mayoría de las histonas por protaminas hace posible un empaquetamiento más compacto de la cromatina, que es necesario para la función normal de los espermatozoides, y puede ayudar a proteger el ADN de los espermatozoides durante el transporte (Carrell, 2011; Ward

y Coffey, 1991). En peces, el genoma del esperma de pez cebra está empaquetado en nucleosomas y unas variantes de las histonas y no en protaminas.

Daño y fragmentación del ADN espermático

Durante las fases finales de espermatogénesis, los sistemas de regulación de reparación del ADN pueden permitir que las células germinales masculinas tengan una mayor posibilidad de daño en el ADN, ya que el mecanismo celular que permite que los espermatozoides se sometan a una apoptosis completa no funciona durante la espermatogénesis. Además, los espermatozoides anormales establecidos originalmente para la eliminación pueden escapar de la apoptosis (Donnelly et al., 2000). Sin embargo, los espermatozoides eyaculados pueden tener daños en el ADN tanto en núcleo como en las mitocondrias. Los orígenes de este daño en el ADN podrían explicarse si se asume que cuando la espermatogénesis se ve interrumpida por el estrés oxidativo, es debido a gametos con la cromatina remodelada defectuosa que es particularmente susceptible al ataque de los radicales libres. Estas células defectuosas tienden a sufrir un inusual acortamiento de la apoptosis para solucionar el problema asociado con las altas cantidades de superóxido generadas por la mitocondria del esperma. Esto conduce a un daño oxidativo notable que finalmente termina en la SDF (*Sperm DNA Fragmentation*) (Aitken y Curry, 2011). Las causas principales del daño en el ADN espermático son el resultado del estrés oxidativo y la apoptosis anormal (Aitken y otros, 1989; Aitken y De Iuliis, 2010; Bui y otros, 2018). Entre las diversas anomalías del ADN, la fragmentación del ADN puede ser la anomalía espermática más frecuente en parejas infértiles. Ahora hay evidencia firme de que un espermatozoide que contiene el ADN fragmentado puede estar vivo, ser móvil, morfológicamente normal y ser capaz de fecundar un ovocito. Además, está demostrado de que los ovocitos tienen cierta capacidad para reparar el ADN dañado del espermatozoide, pero dependiendo del tipo de daño en el esperma y también de la calidad del propio ovocito, como son la inmadurez del ovocito, la edad materna y otros factores externos (González-Marín et al., 2012). Por lo tanto, es importante entender las posibles consecuencias de la fragmentación del ADN espermático para el desarrollo del embrión, la implantación, el resultado del embarazo y la salud de la descendencia, tanto de forma natural como mediante las tecnologías de reproducción asistida (ART).

Diferentes niveles y tipos de daño del ADN espermático

Hay diferentes niveles de defectos de la cromatina de esperma que hay que considerar:

a) Daño a la integridad del ADN en forma de roturas en la cadena simple o doble del ADN.
b) Defectos de las proteínas nucleares que pueden interferir con la trasformación de histonas a protaminas y la subsiguiente compactación del ADN.
c) Anomalías estructurales de la cromatina que causan una configuración modificada de la cromatina terciaria.

Hay varios mecanismos que podrían causar la fragmentación del ADN espermático y / o la integridad de la cromatina dañada.

Causas intrínsecas

a) Estrés oxidativo: la causa principal de la función defectuosa del esperma es el estrés oxidativo, que es un trastorno que refleja un desequilibrio entre la expresión de las especias reactivas del oxígeno (ROS) y la capacidad del sistema biológico para fácilmente eliminar los productos intermedios reactivos (defensas antioxidantes) o para reparar el daño resultante (Agarwal et al., 2014). Los espermatozoides son sensibles al estrés oxidativo porque su membrana plasmática contiene un alto nivel de ácidos grasos poliinsaturados (peroxidación lipídica). La peroxidación de ácidos grasos poliinsaturados genera peróxidos que tienen un efecto perjudicial sobre los espermatozoides, lo que altera la integridad de su ADN. La peroxidación lipídica ocurre cuando los electrones de los lípidos de la membrana plasmática están expuestos a los ROS (Bui et al., 2018). Thaddeus Mann y colaboradores indicaron por primera vez la importancia clínica del estrés oxidativo en la etiología de la función defectuosa del esperma para reconocer la capacidad de la catalasa, como antioxidante, para prevenir la pérdida de movilidad en espermatozoides de hombre (Jones et al., 1979).
b) Apoptosis: cuando las células germinales masculinas realizan su transformación a espermatozoides, pierden su capacidad para sufrir la muerte celular programada. En

lugar de involucrarse en una respuesta apoptótica completa, los espermatozoides pasan de una forma diferente este proceso, llevando a la fragmentación del ADN del núcleo, mientras pueden permanecer con la capacidad de diferenciarse en espermatozoides funcionales maduros con capacidad de fecundar.

c) Anomalías en la remodelación o el empaquetamiento de la cromatina: durante la espermatogénesis cuando las histonas se reemplazan por protaminas, se someten a una hiperacetilación y pronto se reemplazan por protaminas 1 y 2 (P1, P2). Las P1 y P2 se expresan normalmente en una proporción de 1:1 en espermatozoides humanos y proporcionan un empaquetamiento ajustado del ADN del espermatozoide. Se reconoce que las proporciones anormalmente altas y bajas de P1/P2 están asociadas con una mayor fragmentación del ADN del espermatozoide, menores tasas de fecundación, mala calidad del embrión y menores tasas de embarazo (González - Marín et al., 2012).

Causas extrínsecas

Las causas extrínsecas de la fragmentación del ADN espermático y la alteración de la integridad de la cromatina incluyen: estrés oxidativo post-testicular, varicocele, infección bacteriana, edad, abstinencia, temperatura del testículo, reacciones a procedimientos clínicos, medicamentos o vacunas, exposición al entorno químico y tratamientos y procedimientos de tratamiento antirretroviral (González - Marín et al., 2012).

Las especies reactivas del oxígeno (ROS)

Las ROS, también conocidas como radicales libres, tienen al menos un electrón desapareado. Las ROS son agentes oxidantes generados como subproductos del metabolismo oxidativo. Debido al electrón desapareado en la capa externa, forman moléculas altamente reactivas. Las ROS representa una serie de radicales de amplia gama (por ejemplo, ion hidroxilo [OH-], ion superóxido [$O2^{-}$], óxido nítrico [NO], peroxil [RO2], peroxil-graso [LOO] y Tiol [RS-] y moléculas no radicales (oxígeno singlete [$^{-1}O2$], peróxido de hidrógeno [H2O2], peróxido graso [LOOH] y ozono [O3]). Cuando las ROS se producen en grandes cantidades, tienen efectos potencialmente tóxicos en la calidad y

función del esperma. Los espermatozoides, como todas las células que viven en condiciones aeróbicas, se enfrentan constantemente a la paradoja del oxígeno (O2); por un lado requieren O2 para mantener la vida, pero sus metabolitos, como las ROS, pueden alterar negativamente la función del esperma.

Producción de ROS por los espermatozoides

Los espermatozoides generan ROS de dos maneras diferentes:

- Presencia de exceso de citoplasma residual (gota citoplasmática): el defectuoso mecanismo de extrusión citoplasmática y los espermatozoides inmaduros funcionalmente defectuosos podrían afectar la enzima que controla la disponibilidad intracelular de nicotinamida adenina dinucleótido fosfato (NADPH). El NADPH se utiliza como fuente de electrones para alimentar la generación de ROS en los espermatozoides.
- Actividad mitocondrial: el sistema mitocondrial es la fuente clave de producción de ROS en los espermatozoides en los hombres infértiles. La ROS principal generada en los espermatozoides humanos es el O_2^-. Este producto procedente de la reducción de un electrón del oxígeno reacciona de manera secundaria consigo mismo en una reacción de dismutación, para generar H2O2 (Agarwal, 2003).

Producción de ROS por mitocondria

La movilidad de los espermatozoides es el resultado del movimiento flagelar de la cola de los espermatozoides, usando la energía del ATP (adenosina trifosfato) producida en la parte media en las mitocondrias. Las mitocondrias son una fuente importante de producción de ROS en la mayoría de las células en mamíferos. La respiración mitocondrial (cadena respiratoria) es la principal fuente biológica de los radicales aniónicos en condiciones fisiológicas. Davila et al. (2016) observaron que la ATP no se agotaba completamente al inhibir la función mitocondrial y, de igual manera, la movilidad del esperma no se eliminaba por completo. Las ROS no parecen contribuir a reducir la movilidad del esperma. Los inhibidores mitocondriales pueden aumentar o disminuir la producción de ROS según la dosis utilizada.

Técnicas para medir la fragmentación del ADN

Existen varias técnicas para detectar la fragmentación del ADN espermático. A lo largo de los años ha habido un número creciente de pruebas para evaluar y medir los niveles de integridad del ADN. El uso de estas pruebas ha sido impulsado en gran medida por la aparición de ART en humanos y animales. Se han utilizado tanto métodos de ensayos directos como indirectos para medir el daño del ADN del espermatozoide. Los métodos directos más comunes para detectar roturas de ADN incluyen el ensayo TUNEL y el cometa, mientras que los métodos indirectos más comunes para evaluar el daño en el ADN. Unas de las técnicas más usadas para medir el nivel de fragmentación en las muestras de semen es el test de dispersión de la cromatina espermática (SCD). Por su simplicidad y rapidez se ha convertido unas de las técnicas más usadas en laboratorios y clínicas de fertilidad.

Test de dispersión de la cromatina espermática (SCD)

Fernández et al. (2005) desarrollaron un método fácil y rápido para determinar la fragmentación del ADN en células espermáticas en humanos y otras especies animales, llamado test de dispersión de cromatina de espermática (SCD). Los espermatozoides intactos se sumergen en una matriz de agarosa en un portaobjetos, se tratan con una solución ácida para desnaturalizar el ADN que contiene roturas, y luego se tratan con tampón de lisis para eliminar las membranas y proteínas. La matriz de agarosa permite trabajar con espermatozoides no fijados en un portaobjetos en un entorno similar a una suspensión. La eliminación de proteínas nucleares produce nucleoides con un núcleo central y un halo periférico de bucles de ADN dispersos. Esos núcleos de espermatozoides con una fragmentación de ADN elevada producen halos muy pequeños o sin dispersión de ADN, mientras que los espermatozoides con niveles bajos de fragmentación de ADN liberan sus lazos de ADN y forman halos grandes (Fernández et al., 2005). La prueba SCD es un método "simple" en forma de kit. A diferencia de todas las otras pruebas, mide la ausencia de daño en lugar del ADN dañado en el esperma. Sin embargo, la confiabilidad entre observadores de categorizar subjetivamente los halos es una limitación de la técnica de SCD. La evaluación del daño del ADN en esta técnica, al igual que con otros parámetros seminales, es una evaluación subjetiva, pero la extensión real del halo es una variable continua que no se puede reducir simplemente

con una elección. El uso de una matriz de clasificación de diferentes subpoblaciones podría usarse para la definición futura de esta evaluación subjetiva actual.

Los trabajos de investigación de los que consta esta tesis se realizaron con el objetivo de utilizar modelos animales para esclarecer la mejor manera de medir la fragmentación del ADN de manera lo más objetiva posible, en muestras de semen y evaluar el efecto del estrés oxidativo sobre la viabilidad y movilidad de los espermatozoides. Para este fin, se realizaron cuatro experimentos, pero solo tres de ellos se presentan en este documento.

OBJETIVO

El objetivo general de este trabajo fue estudiar las mejores técnicas y métodos para evaluar la fragmentación del ADN del esperma y los impactos del estrés oxidativo en la función del espermatozoide, principalmente en la integridad del ADN. Para este último objetivo, el pez cebra (*Danio rerio*) se utilizó como modelo animal. Sin embargo, se requirieron varios experimentos antes de estudiar específicamente la relación entre el estrés oxidativo y la fragmentación del ADN y su evaluación objetiva. Para ello, se propusieron varios objetivos específicos:

1. Usar el pez cebra (*Danio rerio*) como nuestro primer modelo en infertilidad masculina. El primer paso fue establecer y optimizar las características de la movilidad y la cinética de los espermatozoides a lo largo del tiempo después de la activación del esperma en esta especie en diferentes condiciones de osmolaridad (Experimento 1).

2. Estudiar el efecto de los diferentes grados de estrés oxidativo generado por el H_2O_2 en la movilidad y los parámetros del esperma y cómo estos afectaron a la integridad del ADN del esperma en el pez cebra. Además, se estudió una forma más adecuada de medir el grado de integridad del ADN como variable dicotómica y continua (Experimento 2).

3. En humanos, se estudió las evaluaciones morfométricas de la fragmentación del ADN de la técnica de SCD utilizando dos kits comerciales. Se compararon los resultados utilizando los grupos matemáticos que proporcionan una matriz de clasificación de diferentes subpoblaciones de células reaccionadas con ADN de cabeza de esperma (Experimento 3).

Resultados globales de los tres experimentos

La revisión de los estudios anteriores indicó que hay pocos trabajos que contribuyan a la evaluación de la movilidad y su duración a lo largo del tiempo en el pez cebra, y ninguno sobre la cinética de los espermatozoides y la fragmentación del ADN. Como consecuencia, decidimos comenzar nuestros experimentos analizando la movilidad y la cinética del esperma de pez cebra en las condiciones experimentales. De hecho, la movilidad de los espermatozoides y su duración es uno de los factores más importantes en la fertilidad de los peces, que está claramente relacionado con la osmolaridad del medio de activación. En el presente trabajo, se analiza los efectos de los medios de activación con diferentes osmolalidad y conservación de las muestras sobre la movilidad del espermático y los parámetros cinéticos a lo largo del tiempo, utilizando el sistema CASA (ISAS®) en Danio rerio (Experimento 1). También se estudió el efecto de las muestras pool y de eyaculación individual. El proceso de recolección de semen se llevó a cabo aproximadamente 15 minutos después de que se encendieran las luces. El semen se recogió en animales anestesiados. Los eyaculados se recogieron en un capilar que se colocó en la abertura urogenital. Cada eyaculado fue de aproximadamente 1 µL. Las muestras de semen de machos (de 7 a 9 peces) se agruparon y luego se diluyeron en 100 µl de una solución (HBSS) compuesta de 1,5 g de albúmina de suero bovino (BSA; Sigma-Aldrich, H8264) y se disolvieron 0,1 g de NaCl 100 ml de solución de Hank (Sigma-Aldrich A7906); HBSS tuvo una osmolalidad de aproximadamente 300 mOsm/kg. Los parámetros de la motilidad de los espermatozoides se obtuvieron mediante el sistema CASA (ISAS® v1.2; PROISER S.L., Paterna, España). El microscopio utilizado fue un UOP triocular equipado con un objetivo de contraste de fases negativo (lente Plan 10x PHN), y las grabaciones se realizaron con una cámara digital. Los siguientes valores fueron determinados por el sistema CASA: el porcentaje de espermatozoides móviles totales y progresivos (%), porcentaje de espermatozoides que se mueven con rectitud (STR, %), velocidad curvilínea (VCL, µm/sec), trayectoria de velocidad promedio (VAP, µm/sec), velocidad de línea recta (VSL, µm/sec), oscilación de la trayectoria curvilínea (WOB, relación VAP / VCL, %), linealidad del movimiento (LIN, %), desplazamiento lateral de la cabeza (ALH, µm), y la frecuencia de cruce de latido (BCF, Hz). Los espermatozoides activados con medio de alta osmolaridad

mostraron valores más altos en los parámetros de movilidad y parámetros cinéticas en comparación con el medio de baja osmolaridad. Respecto a los eyaculados individuales o a las muestras pool, aunque se observaron diferencias significativas en algunos parámetros, no se observaron cambios relevantes en la movilidad total ni en los parámetros cinéticos. La movilidad total y la velocidad de los espermatozoides mostraron valores significativamente más bajos después de conservar las muestras durante 24 horas a 4°C. Una vez que se definió el protocolo óptimo de activación de los espermatozoides de pez cebra, estudiamos los efectos del estrés oxidativo del H2O2 en diferentes niveles en los espermatozoides del pez cebra, considerando esta especie como un modelo experimental. Un aumento de las ROS o la disminución de las barreras antioxidantes puede provocar la peroxidación de lípidos de las membranas o el daño del ADN de los espermatozoides. El objetivo de este segundo experimento (Experimento 2) fue estudiar el efecto de los diferentes niveles de estrés oxidativo generado por la incubación con H_2O_2 en la movilidad total, la cinética y la fragmentación del ADN de espermatozoides de pez cebra (*Danio rerio*). Para este proceso, los grupos experimentales se incubaron en 50 µM (Bajo; L) y 200 µM (Alto; H) de H_2O_2, respectivamente, durante 20 min a 4°C. Los parámetros de la movilidad del esperma se analizaron con el sistema CASA. La fragmentación del ADN espermático (SDF) se evaluó utilizando el test de dispersión de la cromatina. La tasa de SDF se evaluó utilizando la técnica de SCDT con el kit comercial Sperm-Halomax (Halotech DNA SL, Madrid, España) diseñado para espermatozoides de peces (Gosálvez et al., 2014). Los espermatozoides fueron teñidos para la evaluación de microscopía de campo claro. Una vez que los portaobjetos se secaron, se tiñeron con el set de tinción Diff-Quick (Medion Diagnostics). Los espermatozoides de los grupos tratados con H_2O_2 mostraron una movilidad más baja que los espermatozoides grupos de control. La movilidad progresiva de los espermatozoides incubados en el grupo H disminuyó rápidamente en comparación con otros grupos. Con respecto a SDF, los grupos control y L tuvieron valores significativamente más bajos que el grupo H (25,0% y 31,6% vs. 48,1% de esperma fragmentado para los grupos C, L y H respectivamente; $P < 0,05$). La movilidad de los espermatozoides, principalmente movilidad progresiva, disminuyó a medida que aumentaba la concentración del tratamiento de H_2O_2, principalmente cuando aumentaba el tiempo después de la activación de los espermatozoides. La SDF aumentó con el aumento de la concentración de H_2O_2. Sin embargo, las medidas del área de halo no coincidieron con la tasa subjetiva de evaluación

de SDF. Se ha demostrado que la fragmentación del ADN es una de las causas de la infertilidad masculina, particularmente relacionada con abortos repetidos, y se han desarrollado diferentes métodos para analizarla. Paralelamente, en el trabajo desarrollado en el modelo del pez cebra, nuestro grupo estaba trabajando también en otras especies, incluida la humana. En el Experimento 3, se evaluaron dos kits comerciales basados en la técnica de SCD (Halosperm® y SDFA) mediante el uso del módulo de fragmentación del ADN de ISAS. v1. Se analizaron siete muestras de semen de voluntarios. Los datos se analizaron mediante Componentes principales (se obtuvieron dos CP) y las subpoblaciones posteriores se identificaron utilizando Halo, Halo / Core Ratio y los datos de las Componentes Principales. Los resultados mostraron que ambos kits están midiendo el nivel de SDF, incluso presentaban diferencias en las características morfométricas de las células reaccionadas. Los resultados de ambos kits fueron significativamente diferentes ($P<0,001$). En cada caso, se obtuvieron cuatro subpoblaciones, independientemente del método de clasificación utilizado. La distribución de las subpoblaciones difería según el kit utilizado. A partir de los datos de las CP, se obtuvo una matriz de análisis discriminante y se obtuvo una buena clasificación a posteriori (97,1% para Halosperm y 96,6% para SDFA). Se utilizó un nuevo enfoque para la clasificación de la SDF, después del análisis de conglomerados (en dos pasos), que reveló que la extensión real del halo es una variable continua que no se puede reducir a un valor discreto por elección. Se demostró que, con estadísticas apropiadas, la clasificación puede basarse en la naturaleza real de las variables. Las cuatro subpoblaciones que encontramos en todos los casos patrullaban la evaluación subjetiva de "no halo", "halo pequeño", "halo medio" y "halo grande" respectivamente, pero sobre la base de datos matemáticos. Esta nueva clasificación se propuso como alternativa a la subjetiva clásica y será muy útil en la futura evaluación de la SDF de espermatozoides en la especie humana.

CONCLUSIONES

- Los espermatozoides activados en el medio de alta osmolaridad mostraron valores de movilidad y parámetros cinéticos más elevados.

- Respecto a muestras individuales o el pool, no se observaron cambios relevantes en la movilidad total o en los parámetros cinéticos.

- La movilidad total y la velocidad del esperma mostraron valores significativamente más bajos tras la conservación a 4ºC.

- La movilidad de los espermatozoides de pez cebra, particularmente la movilidad progresiva, disminuyó a medida que aumentaba la concentración de H_2O_2 en el medio y este efecto perjudicial fue más marcado conforme se incrementaban los tiempos de incubación.

- Los niveles subjetivos de SDF aumentaron a medida que aumentaban las concentraciones de H_2O_2 en el medio, pero las mediciones del área del halo no coincidían con la evaluación subjetiva de la tasa de SDF.

- Los diferentes kits basados en el principio de SCD ofrecen resultados diferentes, siendo todos ellos representativos de SDF en muestras humanas.

- Se observaron cuatro subpoblaciones basadas en datos morfométricos obtenidos de muestras preparadas con el nivel SDF definido por Halosperm y SDFA y se pueden utilizar reemplazando a la evaluación subjetiva anterior en humanos.