TESIS DOCTORAL

Análisis de la fragmentación del ADN del espermatozoide canino mediante el Test de Dispersión de la Cromatina Espermática

Analysis of the DNA fragmentation on canine sperm using the Sperm Chromatin Dispersion test

Mª Teresa Urbano Luque

Córdoba, 2017

TITULO: *Análisis de la fragmentación del ADN del espermatozoide canino mediante el Test de Dispersión de la Cromatina Espermática*

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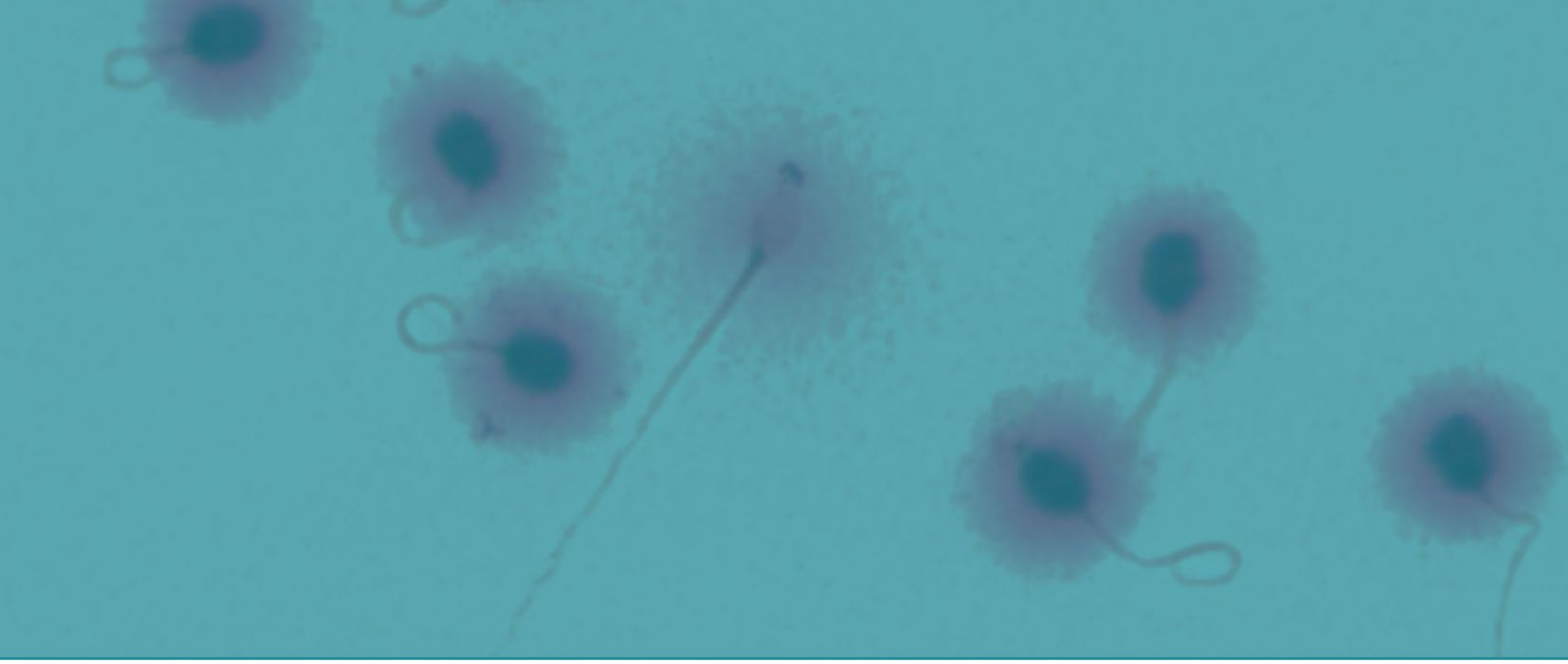

UNIVERSIDAD DE CÓRDOBA
FACULTAD DE VETERINARIA
DEPARTAMENTO DE MEDICINA Y CIRUGÍA ANIMAL

FACULTAD DE VETERINARIA

Departamento de Medicina y Cirugía Animal

"ANÁLISIS DE LA FRAGMENTACIÓN DEL ADN DEL ESPERMATOZOIDE CANINO MEDIANTE EL TEST DE DISPERSIÓN DE LA CROMATINA ESPERMÁTICA"

"Analysis of the DNA fragmentation on canine sperm using the sperm chromatin dispersion test"

Memoria para optar al grado de Doctor presentada por:

Mª Teresa Urbano Luque

Bajo la dirección de los Doctores:

Manuel Hidalgo Prieto

Jesús M. Dorado Martín

Isabel Ortiz Jaraba

Córdoba, 2017

TÍTULO DE LA TESIS: Análisis de la fragmentación del ADN del espermatozoide canino mediante el test de dispersión de la cromatina espermática.

DOCTORANDO/A: MARIA TERESA URBANO LUQUE

INFORME RAZONADO DEL/DE LOS DIRECTOR/ES DE LA TESIS

D. Manuel Hidalgo Prieto, D. Jesús M. Dorado Martín y Dña. Isabel Ortiz Jaraba, profesores del Departamento de Medicina y Cirugía Animal de la Facultad de Veterinaria de la Universidad de Córdoba

INFORMAN:

Que el trabajo de tesis presentado por Dña. Mª Teresa Urbano Luque, titulado *"Análisis de la fragmentación del ADN del espermatozoide canino mediante el test de dispersión de la cromatina espermática"* ha sido realizado bajo nuestra dirección y cumple con los artículos 24 y 35 de la norma reguladora de los Estudios de Doctorado de la Universidad de Córdoba para su presentación como compendio de publicaciones, así como para obtener la mención internacional.

Por todo ello, se autoriza la presentación de la Tesis Doctoral.

Córdoba, 30 de mayo de 2017

Firma de los directores

Fdo.: Manuel Hidalgo Prieto Fdo.: Jesús M. Dorado Martín Fdo.: Isabel Ortiz Jaraba

European/International Mention in the Doctorate Degree

Certificate of stay

I hereby confirm that Mr. / Mrs Maria Teresa Urbano Luque has steadily stayed at Department of Clinical Sciences, Swedish University of Agricultural Sciences from 01/09/2014 to 30/11/2104 and has successfully developed research in Assessment of sperm DNA fragmentation using the sperm chromatin structure assay (SCSA) and sperm chromatin dispersion test (SCDt) on frozen epididymal sperm in dog that is directly related with his/her PhD thesis in Study of the sperm DNA fragmentation in dog using the sperm chromatin dispersion test.

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Institutional Stamp

Signed in Uppsala, 1st of Dec, 2014

INFORME DOCTORES INTERNACIONALES
TESIS DOCTORAL

REFEREE REPORT ON THE PhD TESIS PRESENTED
IN THE UNIVERSITY OF CÓRDOBA (SPAIN)
BY MARÍA TERESA URBANO LUQUE

TITLE OF THE THESIS:	ANALYSIS OF THE DNA FRAGMENTATION ON CANINE SPERM USING THE SPERM CHROMATIN DISPERSION TEST

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for	This thesis meets the requirements for presentation as an oral dissertation	YES	NO
		x	

Rating	Originality	Scientific /technical merit	Planning /methodology
Outstanding	x	x	x
Excellent			
Very Good			
Good			
Sound			
Defficient			

COMMENTS (Please use additional sheets, if necessary):

DNA defragmentation index is a very valuable tool for dog semen quality interpretation. Even normally it is not frequently used test for dog semen assessment it seems to have a very big impact on fertilty rate in this species.
In this thesis it was found that long term storage of dog semen significantly increased DFI, but what was surprised, the semen cryopreservation did not change DFI.
Moreover fourth paper summarized that different tests for DNA status in sperm could show us different outcomes resulting from different action of stains and techniques.
This thesis book presents a series of relevant points for what the doctoral candidate deserves to proceed to the oral presentation.

1. Format
The current thesis is clear and well organized. The title and the abstract reflect the contents and the introduction clearly states the problem being investigated and provides an adequate background. The present thesis consists: summary, introduction, objectives, chapters with papers, conclusions, references, quality indicators and other scientific contributions derived directly from the Doctoral Thesis. I wish that the last two chapters were not in English.
Despite the lack of a general discussion, each paper contains discussion section that strongly support the results collected from the different experimental designs carried out. It would be very difficult to construct one compact discussion for each experiment performed in these four papers.

2. Methodology

The materials & methods sections in each paper are described in details allowing repeat such experiments. Techniques used for sperm quality includes sperm motility parameters (Sperm Class Analyzer), concentration (photometrically), sperm morphology (Computer-assisted sperm morphometry analysis (CASMA)), sperm DNA fragmentation (SCDt, Hallomax, Hallotech DNA SL), Sperm chromatin structure (SCSA, flow cytometry).

Statistical procedures are advanced and adequate for the proper analysis of the data resulted from the different experimental designs. There were used bland Atman test, correlations, ANOVA, t-Student test and the intraclass correlation coefficient which provide the accurate analysis of the experimental data.

3. Publications

This thesis was divided on four thematic sections, which each consists very valuable results.

The division was very logical and contained paper:

1. DNA integrity of canine spermatozoa during chill-storage assessed by the sperm chromatin dispersion test using bright field or fluorescence microscopy
2. Effect of cryopreservation and single layer centrifugation on canine sperm DNA fragmentation assessed by the sperm chromatin dispersion test
3. Identification of sperm morphometric subpopulations in cooled-stored canine sperm and its relation with sperm DNA integrity
4. Comparison of DNA fragmentation of frozen-thawed epidydimal canine sperm using SCSA and SCDt

A relevant aspect highlighted in the first paper that DFI of dog sperm increased after liquid storage was also confirmed in the third publication. Assessment the sperm DNA integrity using Sperm-Halomax detects sperm damage earlier than other routine sperm parameters. Moreover this test could be easly used with light microscopy and also with fluorescence.

In the secon paper no effect of cryopreservation was observed on dog sperm DNA fragmentation. Additionally, SLC-selection using Androcoll-C improved longevity of frozen-thawed sperm DNA.

The third publication concerns the subject of the role of sperm head morphometry on the prediction of freezability. Taking into consideration that sperm head morphometric parameters may reflect chromatin organization, four morphometric sperm subpopulations in dogs with different response to cooling process were detected. Although the population with small and rounded sperm heads increased during cool storage, and there was a moderate positive correlation between sP3 and DNA fragmentation, it could not be concluded that sP3 only contains sperm with fragmented DNA.

The fourth paper is not published yet, but its content is interesting. Comparison of the sperm DNA fragmentation of frozen-thawed epidydimal canine sperm using the SCSA and SCDt tests was presented. Although SCSA and SCDt can evaluate the changes in the sperm DNA fragmentation dynamics of frozen-thawed epididymal dog sperm, they generate different information on sperm DNA fragmentation.

4. Scientific value

Two of the journals where the manuscripts have been published are included in the first quartile and one published and one sent are in the second quartile of Agriculture, Dairy and Animal Science and Veterinary Sciences. Moreover the Impact Factors of presented journals are high. Therefore, it confirms a very high scientific relevance of the of the present thesis book.

Additional abstracts presented during international meetings confirm the significant scientific value of the present thesis book.

For all the evidence provided, I can recommend the PhD candidate, María Teresa Urbano Luque, for the PhD degree.

DATE: Wroclaw, 14th May 2017

SIGNATURE: Agnieszka Partyka

INFORME DOCTORES INTERNACIONALES
TESIS DOCTORAL

REFEREE REPORT ON THE PhD TESIS PRESENTED
IN THE UNIVERSITY OF CÓRDOBA (SPAIN)
BY MARÍA TERESA URBANO LUQUE

TITLE OF THE THESIS:	ANALYSIS OF THE DNA FRAGMENTATION ON CANINE SPERM USING THE SPERM CHROMATIN DISPERSION TEST

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for		YES	NO
	This thesis meets the requirements for presentation as an oral dissertation	X	

Rating	Originality	Scientific /technical merit	Planning /methodology
Outstanding	X	X	X
Excellent			
Very Good			
Good			
Sound			
Defficient			

COMMENTS (Please use additional sheets, if necessary):

Sperm chromatin integrity and DNA structure are ones of the most important features conditioning sperm cell fertilizng ability. Some tests assessing DNA/chromatin sperm structure have been developed such as SCSA, acridine orange AO labelling, COMET Assay and TUNEL Assay. All these tests necessitate the specialised equipment/laboratory and skilled technicians. There is great demand for establishement, validatiation as well as assessment of the relibility of more simple test assessing DNA/chromatin structure. Author used for this puspose very promising Sperm Chromatin Dispersion Test SCDt .
It should be emphasized, that in spite of the fact of performing of many research studies and establishement of reference values for DNA Fragmentation Index in human andrology, in animal andrology the normal and abnormal values of chromatin structure status should be elucidated. On the other hand there are scarce data on changes of chromatin structure in chilled and frozen-thawed ejaculated and epididymal dog sperm. From this point of view the work of Candidate meets the demand from the side of basic and applied science.

The book of Thesis is clearly organized and meets all requirements demanded in research. The author present clearly written abstract, 4 publications published/submitted (3 published and 1 submitted) in per-reviewed JCR journal of high reputation and conclusions. Publications are focused on assessment of sperm chromatin

structure in chilled semen, in cryopreseved semen, in sperm morphometric subpopulations and in epididymal sperm. Candidate commented the results obtained and clearly concluded them.

This thesis book presents a series of relevant points for what the doctoral candidate deserves to proceed to the oral presentation.

1. Format
The thesis is presented in a clear and well-organize manner. The text hierachy is clear and easy to understand. It makes easy to follow the objective through the thesis book. Each of four publications contains the discussion chapter which deeply comment results obtained and compares these results with literature data. Thereafter author concluded all the publications which makes the reception of thesis book more easy.

2. Methodology
The methods of semen assessment was chosen adequately. The computer assisted sperm analysis CASA was used in all experiments. CASA assessments consisted of regular motility assessment but also of analysis of subpopulations according to morphometric dimensions. The SCDt and SCSA tests were used for chromatin integrity assessment. Statistical analysis consisting of ANOVA with post hoc tests and analysis of non parametric data with the use of such tests as Kruskal-Wallace were absolutely correct. The analysis of subpopulations divied according to morphometric data represents a good example of the accurate analysis of the experimental data.

3. Publications
Sperm Chromatin Dispersion tests was used in chilled dog semen (first paper), in frozen-thawed semen (second publication), in morphometric subpopulations of sperm cells (third publication) and in epididymal dog sperm cells (fourth publication-submitted). First publication confirmed that Halomax test based on the SCDt, can be used to assess the sperm DNA integrity of chilled-stored canine semen in bright field and fluorescence microscopy. Interesting is, that sDFi increased after 48 hours of chill storage, which could detect sperm damage earlier than motility drop is observed. In the second publication no effect of cryopreservation was observed on values of dog sperm DNA fragmentation when SCDt was used. SLC-selection using Androcoll-C improved longevity of frozen-thawed sperm DNA assessed after 24 hours by SCDt. In fourth paper four sperm morphometric subpopulations were identified. Only sP3 (small and rounded sperm heads) increased progressively after 72h of cool-storage. This population may be therefore a possible indicator of sperm damage. There was a positive correlation between sP3 and DNA fragmentation. SCSA and SCDt can evaluate the changes in the sperm DNA fragmentation dynamics of frozen-thawed epididymal dog sperm. Additionally it was observed that both these tests yielded different information on sperm DNA fragmentation. All above results and conclusions are interesting from the point of view of basic and applied science. All papers put the novel on the field of animal reproduction. The results appear to be interesting not only for researchers but also for clinical andrologists.

4. Scientific value
The journals where the manuscripts have been published are recognised as having high scientific reputation. Theriogenology, Animal Reproduction Science and Reproduction in Domestic Animals are leading journals on the field of animal repsordution with high impact and citation indices. Additonaly Reproduction in Domestic Animals is the official organ of one of the most important organizations in veterinary reproduction community i.e. ESDAR and EVSSAR. It is clear the scientific relevance of the papers published. It should be noted this thesis resulted also in communications presented in very prestigious international meetings such as ESDAR Conferences and IETS. It confirms the highly significant scientific value of the present thesis book.

Concluding, I can strongly recommend the PhD candidate, Maria Teresa Urbano Luque, for the PhD degree.

DATE: 14.05.2018

SIGNATURE:

INFORME DOCTORES INTERNACIONALES
TESIS DOCTORAL

REFEREE REPORT ON THE PhD TESIS PRESENTED
IN THE UNIVERSITY OF CÓRDOBA (SPAIN)
BY MARÍA TERESA URBANO LUQUE

TITLE OF THE THESIS:	ANALYSIS OF THE DNA FRAGMENTATION ON CANINE SPERM USING THE SPERM CHROMATIN DISPERSION TEST

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	YES	NO
This thesis meets the requirements for presentation as an oral dissertation	x	

Rating	Originality	Scientific /technical merit	Planning /methodology
Outstanding	x	x	x
Excellent			
Very Good			
Good			
Sound			
Defficient			

COMMENTS (Please use additional sheets, if necessary):

In this Thesis the assessment of DNA fragmentation by a chromatin dispersion test has been employed in fresh, chilled and frozen-thawed canine spermatozoa, including epydidimal cryopreserved samples. The use of bright field and fluorescence microscopy has also been compared for the evaluation chromatin dispersion. Only some preliminary data on the use of this method to detect sperm damage in fresh canine spermatozoa were present beforse the beginning of this Thesis. Thus, the subject can be considered original. The results of this Thesis show that the employied kit, Sperm-Halomax, can function both under bright field and fluorescence microscopy, and that sperm DNA degradates during cooling and freezing procedures. Moreover, as seen in other species, Androcoll-C is able to select the best frozen-thawed sperm and thus increased post-thaw unfragmented DNA. A correlation between the proportion of canine sperm with small, rounded heads and the proportion of those with DNA fragmentation was found, although it was not possible to state that those were the same cells. Finally, two more different classes of morphology of sperm DNA fragmentation were seen in epididymal spermatozoa, suggesting that these sperm may behave differently. These results have the merit to add a technique to those available for dog semen evaluation, and to add new scientific knowledge on the DNA changes occurring in different steps of sperm maturation and cryopreservation. The methods were adequate and innovative in the choiche for DNA testing, and statistical analyses were excellently employed. Finally, the Thesis includes 3 full papers published and 1 submitted in important peer reviewed journals and is thus available to the scientific community.

DATE: Pisa, May 12th, 2017

SIGNATURE: Alessandra Rota

TESIS DOCTORAL COMO COMPENDIO DE PUBLICACIONES

1. M Hidalgo, M Urbano, I Ortiz, S Demyda-Peyras, MR Murabito, MJ Gálvez, J Dorado (2015). *"DNA integrity of canine spermatozoa during chill-storage assessed by the sperm chromatin dispersion test using bright field or fluorescence microscopy"*. Theriogenology 84: 399-406.

2. M Urbano, J Dorado, I Ortiz, JM Morrell, S Demyda-Peyras, MJ Gálvez, L. Alcaraz, L Ramírez, M Hidalgo (2013). *"Effect of cryopreservation and single layer centrifugation on canine sperm DNA fragmentation assessed by the sperm chromatin dispersion test"*. Animal Reproduction Science 143: 118-125.

3. M Urbano, I Ortiz, J Dorado, M Hidalgo (2017). *"Identification of sperm morphometric subpopulations in cooled-stored canine sperm and its relation with sperm DNA integrity"*. Reproduction in Domestic Animal 52 (3): 468–476.

4. I Ortiz, M Urbano, J Dorado, JM Morrell, E Al-Essawe, A Johannisson, M Hidalgo. *"Comparison of DNA fragmentation of frozen-thawed epididymal canine sperm using SCSA and SCDt"*. Enviado a Animal Reproduction Science.

ACKNOWLEDGMENTS

AGRADECIMIENTOS

AGRADECIMIENTOS

Es difícil resumir casi cinco años dedicados a éste proyecto que a día de hoy ve su fin. Han sido muchos momentos, situaciones y experiencias tanto buenas como malas que han hecho de éste trabajo una pequeña montaña rusa. Son muchas las personas con las que a lo largo de éstos casi cinco años he hablado, he compartido y he experimentado mis vivencias acerca de la Tesis. También muchos consejos, ánimos, penas y alegrías compartidas con todas y cada una de estas personas; y en parte a todos ellos les debo ponerle fin a éste proyecto.

- Empezando por mi director, Manuel Hidalgo, o para mí Manolo. A él le debo el haber empezado en el tema de la investigación. Me animó a comenzar con la Tesis Doctoral, a pesar de no contar con financiación, ni becas, ni trabajo en un principio. Después hizo posible que pudiese continuar haciéndola desde la distancia, no siendo fácil, pero si posible. He aprendido mucho en éstos casi cinco años, bien de ti o muchas veces contigo. Por esto y por todos los momentos vividos dentro y fuera del despacho, porque no solo has sido director sino también amigo, sólo decirte MIL GRACIAS por tu apoyo, tu ayuda y por permitir que esto llegue a su fin.

- A Jesús Dorado, mi segundo director. Desde el principio has sido un apoyo y un referente a seguir. Siempre has estado dispuesto a explicarme lo que he necesitado, a enseñarme lo que no he sabido y a mantener el orden dentro del caos. GRACIAS POR TODO.

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- A los compañeros de Uppsala, Essraa, Denise y en especial a Celina, porque me ofrecieron su casa y me acogieron haciéndome sentir como en casa, y eso es algo muy valorado cuando éstas sólo y lejos. Por supuesto a Jane Morrell y Anders Johannisson porque hicieron posible mi estancia internacional y permitieron que pudiera realizar mi trabajo sin ningún tipo de impedimento. Por supuesto no puedo olvidar a mis compañeros de batalla en Uppsala, a Enrique y Sara porque se convirtieron en mi pequeña familia, con la que poder evadirme y compartir el día a día. Siempre os estaré agradecida.

- A mi familia, mis padres y hermana. Me dieron los recursos que en un inicio no tuve, y si hoy termino éste proyecto es gracias a ellos. Siempre me han apoyado y nunca han dudado de mi perseverancia y esfuerzo. Han sido muchos los momentos en los que me he planteado tirar la toalla, pero siempre han estado animándome y

recordándome que “con actitud positiva, todo se consigue” (sobre todo mi hermana, la positiva de la familia). GRACIAS por estar ahí y por convertirme en lo que soy hoy, para bien y para mal.

- A todos aquellos que me habéis animado en momentos de debilidad, Marina, Flori, Paula, Antonio Pablo, Belén, Eva, Juan Carlos y Mª Ángeles, a mis compis de trabajo en Granada, y a todos y cada uno de aquellos que en algún momento habéis tenido unas palabras de ánimo y apoyo.

- A Alberto López Jordán, por ayudarme a ponerle cara a éste trabajo. No es fácil crear una portada que represente todo lo que engloba esta Tesis, y tú lo ha conseguido. Muchas gracias por haber ocupado parte de tu tiempo.

- Por último, a mi apoyo incondicional, mi compañero, amigo y pareja, José Carlos. GRACIAS por tu paciencia, tu ayuda, tu apoyo y por aguantarme mis buenos y malos momentos durante todo éste tiempo. GRACIAS por estar siempre ahí y por recordarme constantemente que esto era una carrera de fondo y que todo esfuerzo tiene su recompensa.

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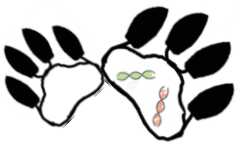


SUMMARY

SUMMARY

Sperm quality has been traditionally evaluated using subjective parameters included in the spermiogram. Additionally, the evaluation of other parameters, such as sperm DNA integrity, has explained subfertility problems in men with normal values of routine sperm parameters but higher sperm DNA fragmentation. Different techniques have been described for evaluating sperm DNA integrity, such as TUNEL (Terminal dUTP Nick-End Labeling), acridine orange test, SCSA (Sperm Chromatin Structure Assay) or comet assay. However, some of these techniques are laborious or required expensive equipments. For this reason, the Sperm Chromatin Dispersion test (SCDt) was developed. This is an easy and cheap method that has been used on different species, including human beings. However, only a preliminary study on fresh canine semen was performed before this Doctoral Thesis. Therefore, the aim of this Thesis was to develop the SCDt for canine sperm DNA fragmentation analysis as a useful tool for different assisted reproduction techniques, such as sperm cooling and freezing, sperm selection and computer-assisted sperm morphometry analysis. For that purpose, four studies were performed.

The objective of the first study was to evaluate the effect of chill storage on canine sperm DNA fragmentation assessed by the sperm chromatin dispersion test (SCDt) using bright-field microscopy with Wright solution or fluorescence microscopy with propidium iodide. Dog semen samples were cooled at 5°C for 96 hours. Firstly, the relationship and agreement between the results obtained with both staining methods were analyzed. Good agreement and no significant differences between values were

obtained with both staining procedures were observed. Secondly, the effect of cool storage for up to 96 hours was assessed on sperm motility parameters and DNA fragmentation indexes. DNA fragmentation significantly increased after 48 hours of cool storage; in contrast, progressive sperm motility decreased after 96 hours. In conclusion, the Sperm-Halomax kit, developed for canine semen and based on the sperm chromatin dispersion test, can be used accurately under bright-field or fluorescence microscopy to assess the sperm DNA integrity of canine semen during cool storage. The sperm DNA fragmentation index increased after 48 hours of chill storage, thereby detecting sperm damage earlier than other routine sperm parameters, such as sperm motility.

In the second study, the effect of freezing and thawing on dog sperm DNA fragmentation index (sDFI) using the sperm chromatin dispersion test (SCDt) was assessed. Moreover, it was determined whether or not the sperm selection by single layer centrifugation (SLC) using Androcoll-C improved sperm DNA longevity in SLC-selected frozen-thawed dog semen samples. Semen samples were collected from 4 dogs and the ejaculates were pooled and cryopreserved following a standard protocol. sDFI was analyzed immediately, before freezing and after thawing, showing no significant differences between fresh and frozen-thawed semen samples. In addition, frozen-thawed semen samples were processed or not by SLC using Androcoll-C. Thereafter, sDFI were assessed after 24 hours of *in vitro* incubation at physiological temperature (38°C). The results showed low values of sDFI in SLC-selected sperm in comparison to unselected samples. In conclusion, no effect of cryopreservation was observed on baseline values of dog sperm DNA fragmentation. Additionally, SLC

selection using Androcoll-C improved longevity of frozen-thawed sperm DNA assessed by the SCDt.

The aims of third study were to identify different morphometric subpopulations in cooled-stored canine sperm and their patterns of distribution during cool-storage for up to 240 hours, determining whether or not morphometric sperm subpopulations (sP) were related to sperm DNA integrity. Semen samples were collected from 4 dogs and the ejaculates were pooled and cooled at 5°C during 240 hours. Morphometric parameters were analyzed by computer-assisted sperm analysis (CASA) and sDFI using the sperm Halomax test. Four morphometric sperm heads subpopulations were identified: sP1 (large and rounded), sP2 (large and elongated), sP3 (small and rounded) and sP4 (small and elongated), and the distribution along the cool storage was analyzed. SP1 was the most predominant subpopulation for up to 72 hours and thereafter sP3 increased progressively. sDFI increased after 48 hours of cool-storage. Although sP3 showed a positive correlation with sDFI, and both increased over time, it could not be ensured that only the sperm with fragmented DNA are accumulated in sP3. In conclusion, sP3 and DNA fragmentation increased progressively during cool-storage, becoming possible indicators of sperm damage. However, it cannot be concluded that sP3 only contains sperm with fragmented DNA.

The aim of the fourth study was to compare the sperm DNA fragmentation of frozen-thawed epididymal canine sperm using the SCSA and SCDt. Epididymis from neutered dogs were minced and incubated in a Tris-based extender. The recovered sperm were slowly cooled, frozen and stored in liquid nitrogen tanks. After thawing, each replicate was incubated at 38°C for 24 hours. sDFI was assessed by SCSA and SCDt

at 0, 3, 6 and 24 hours of incubation and compared within treatments. The relationship and agreement between techniques were evaluated. Both techniques showed a significant increase of DNA fragmentation after 24 hours of incubation. Moderate correlation ($r=0.65$; $P<0.01$) but poor agreement (ICC=0.451; $P>0.05$) was found between SCSA and SCDt. Four halo morphologies were found after 24 hours of incubation using the SCDt: unfragmented DNA with a small halo, fragmented DNA with large halo and two new halo presentations never described before for dog sperm: "receding sperm" with a disappearing halo and "bald sperm" without halo of chromatin dispersion around the core. Bald sperm are not described by the manufacturer and they are similar to unfragmented sperm, which could lead to erroneous results in the SCDt. Further studies with different incubation periods and considering the new morphologies described in this study should be performed. In conclusion, although SCSA and SCDt can evaluate the changes in the sperm DNA fragmentation dynamics of frozen-thawed epididymal dog sperm, they yielded different information on sperm DNA fragmentation.

SUMMARY

RESUMEN

RESUMEN

La calidad espermática ha sido tradicionalmente evaluada mediante parámetros subjetivos incluidos en el espermiograma. Además, la evaluación de otros parámetros, como la fragmentación del ADN espermático ha explicado problemas de fertilidad en hombres, que a pesar de presentar valores normales en los parámetros espermáticos rutinarios, presentaban un alto índice de fragmentación del ADN espermático (sDFI). Existen diferentes técnicas para valorar la integridad del ADN espermático, como el ensayo TUNEL (Marcado del extremo libre por la desoxi-transferasa terminal o dUTP), el test de naranja de acridina, el SCSA (Análisis de la estructura de la cromatina espermática) y el ensayo cometa. Algunas de éstas técnicas requieren de equipos caros o de métodos laboriosos. Por esta razón se creó el SCDt (test de dispersión de la cromatina espermática). Es un método fácil, económico y que ha sido empleado en varias especies animales, incluido el hombre. Sin embargo, en el perro sólo se desarrolló un estudio preliminar en semen fresco, antes de dar comienzo a esta Tesis Doctoral. El objetivo de esta Tesis fue desarrollar el test de dispersión de la cromatina espermática para analizar la fragmentación del ADN en el perro, así como para ver su utilidad en diferentes técnicas de reproducción asistida como son la refrigeración y la congelación de semen, la selección de espermatozoides y el análisis morfométrico del espermatozoide. Para ello se realizaron cuatro estudios:

En la primera publicación estudiamos la integridad del ADN del espermatozoide refrigerado de perro mediante el SCDt, usando microscopía de campo claro con la tinción de Wright y de fluorescencia con el ioduro de propidio. Para ello, las muestras

espermáticas fueron refrigeradas a 5°C durante 96 horas. En primer lugar, se determinó la correlación y concordancia entre los resultados obtenidos, observando buena concordancia y asusencia de diferencias significativas entre los valores obtenidos con ambas técnicas. Posteriormente, se valoró el efecto de la refrigeración durante 96 horas sobre el movimiento espermático y el sDFI. El sDFI aumentó significativamente ($P<0,05$) a las 48 horas de refrigeración, en comparación con el movimiento progresivo que comenzó a decrecer a las 96 horas. En conclusión, el test Halomax®, creado específicamente para semen canino, y basado en el SCDt, puede usarse para valorar la integridad del ADN espermático del semen refrigerado de perro utilizando tanto microscopía de campo claro como de fluorescencia. El sDFI aumentó a las 48 horas de refrigeración, por lo que podría detectar el daño espermático de forma más temprana que otros parámetros espermáticos de rutina, como el movimiento espermático.

En la segunda publicación valoramos el efecto de la congelación sobre la fragmentación del ADN del espermatozoide canino mediante el SCDt, y valoramos si la centrifugación en una sola capa usando Androcoll-C mejoraba la longevidad del ADN en las muestras seleccionadas y congeladas de semen de perro. Las muestras espermáticas recogidas de 4 perros, se mezclaron y sometieron a un protocolo estándar de congelación. El sDFI se analizó inmediatamente antes y después de la congelación, no encontrando diferencias estadísticamente significativas ($P>0,05$) entre el semen fresco y el congelado. Además, las muestras de semen congelado fueron sometidas a un proceso de selección espermática mediante centrifugación coloidal con Androcoll-C, valorándose el sDFI tras 24 horas de incubación a temperatura fisiológica (38°C). El semen seleccionado tras la congelación mostró menor sDFI que él no seleccionado. En

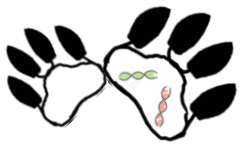
conclusión, el SCDt no detectó ningún efecto en los valores basales de fragmentación del ADN espermático del perro tras la criopreservación. La centrifugación coloidal de una sola capa usando Androcoll-C mejoró la longevidad del esperma congelado de perro, en base a la integridad del ADN espermático valorada con el SCDt.

El objetivo de la tercera publicación fue identificar diferentes subpoblaciones morfométricas, así como su distribución en el esperma refrigerado de perro durante 24 horas, determinado si esas subpoblaciones están o no relacionadas con la integridad del ADN espermático valorado con el SCDt. Se analizaron diferentes parámetros morfométricos empleando un sistema CASA, así como la fragmentación del ADN espermático (sDFI) mediante el SCDt. Cuatro subpoblaciones morfométricas (sP) fueron identificadas en el semen refrigerado de perro: sP1 (espermatozoides grandes y redondos), sP2 (espermatozoides grandes y alargados), sP3 (espermatozoides pequeños y redondos) y sP4 (espermatozoides pequeños y alargados) y se analizó su distribución a lo largo del tiempo de refrigeración. sP1, seguida de sP4 y sP3, fueron las subpoblaciones más abundantes hasta las 72 horas; sin embargo a partir de las 96 horas, sP1 y sP4 disminuyeron y sP3 aumentó progresivamente hasta las 240 horas. sP2 se mantuvo más o menos constante a lo largo de las 240 horas. En conclusión, se identificaron cuatro subpoblaciones morfométricas en el esperma refrigerado de perro, pero sólo la sP3 (espermatozoides con cabezas pequeñas y redondas) aumentó progresivamente tras 72 horas de refrigeración, siendo por lo tanto un posible indicador del daño espermático durante la refrigeración. Aunque existe una correlación positiva entre la sP3 y la fragmentación del ADN espermático, no es posible concluir que la subpoblación 3 solamente incluya espermatozoides con el ADN fragmentado.

El objetivo de la cuarta publicación fue comparar la fragmentación del ADN del esperma congelado de epidídimo de perro usando el SCSA y el SCDt. Para ello, se emplearon epidídimos de perros castrados, que fueron troceados e incubados en diluyente TRIS. El esperma obtenido fue congelado y almacenado en nitrógeno líquido. Después de la descongelación, cada replica fue incubada a 38°C durante 24 horas. Se valoró el índice de fragmentación del ADN espermático mediante SCSA y SCDt a las 0, 3, 6 y 24 horas de incubación y se compararon las técnicas. Además, se valoró la relación y el grado de concordancia entre dichas técnicas. Ambas técnicas mostraron un aumento significativo de la fragmentación del ADN espermático tras 24 horas de incubación. También se encontró una correlación moderada ($r=0,65$; $P<0,01$) y una baja concordancia ($ICC=0,451$; $P>0,05$) entre el SCSA y el SCDt. Asimismo, se identificaron 4 tipos de halo espermático según su morfología, usando el SCDt tras 24 horas de incubación. Por un lado, las dos morfologías habituales de espermatozoides con ADN no fragmentado, mostrando un halo de dispersión de la cromatina pequeño o con ADN fragmentado con un halo grande; y, por otro dos nuevas morfologías no descritas anteriormente en espermatozoides de perro: espermatozoides fragmentados con el halo desplazado, en proceso de desaparición, y espermatozoides "calvos", sin halo de cromatina espermática alrededor de la cabeza, que ha sido desplazado completamente. Los espermatozoides calvos no están descritos por el fabricante y son espermatozoides fragmentados que han perdido completamente el halo, mostrado una apariencia muy similar a los espermatozoides no fragmentados, lo cual podría llevar a errores en la interpretación de los resultados del SCDt. En este sentido, consideramos que sería recomendable incorporar el análisis de las nuevas morfologías descritas en este trabajo, en futuros estudios que evalúen la fragmentación del ADN canino a diferentes tiempos

de incubación. En conclusión, aunque el SCSA y el SCDt pueden evaluar los cambios dinámicos en la fragmentación del ADN del espermatozoide congelado de epidídimo de perro, ambas técnicas ofrecen una información diferente sobre la fragmentación del ADN del espermatozoide del perro.

INTRODUCCIÓN



INTRODUCTION

INTRODUCTION

Nowadays, the cryopreservation of canine sperm is an important tool to preserve genetic material from valuable animals, as well as for the preservation of endangered canids (Zindl et al. 2006). Sperm quality has been traditionally evaluated using subjective parameters included in the spermiogram (Colenbrander et al. 2003). Although, these parameters have been correlated with fertility, they do not provide information about sperm DNA integrity (González-Marín et al. 2012). In this sense, sperm DNA integrity has been proposed as an important index of potential fertility evaluated *in vitro* (Nijs et al. 2011). In addition, the evaluation of human sperm DNA integrity has explained subfertility in men with normal values of routine sperm parameters but higher sperm DNA fragmentation (Giwercman et al. 2003). For this reason, sDFI should be considered an important parameter to assess with the classic spermiogram.

Different techniques have been described for evaluating sperm DNA integrity, such as TUNEL (Terminal dUTP Nick-End Labeling), acridine orange test, SCSA (Sperm Chromatin Structure Assay) or comet assay (Chohan et al. 2006, Evenson 2016). However, some of these techniques are laborious or required expensive equipments. For this reason, the Sperm Chromatin Dispersion test (SCDt) was developed as a new easier and cheaper method to assess sDFI (Fernández et al. 2003). This technique is based on the inclusion of spermatozoa in a gel matrix, applying a high-salt low-pH treatment (including reducing agents to break disulfide bonds in the chromatin). Then, the sample is analyzed by bright-field or fluorescence microscopy, using Wright solution or DNA-specific binding fluorochrome (DAPI, propidium iodide,

Ethidium Bromide or Synergy Brand related fluorochromes) respectively. The analysis of a minimum number of three hundred spermatozoa per sample is needed to obtain the percentage of DNA fragmentation. Depending on the commercial kit used (presence or not of a denaturation treatment in order to optimize the test for different species, according to the authors) the halo of dispersed chromatin indicates either good or bad condition of the sperm DNA. In the case of canine sperm when the SCDt is performed, sperm with fragmented DNA produce large and spotty halos of dispersed chromatin around the nuclear core (arrows, Figure 1). Sperm nuclei with unfragmented DNA produce very small and compact halos of chromatin dispersion. As described above, the relative size of the sperm halos may be easily visualized under bright-field or fluorescence microscopy (Figure 1).

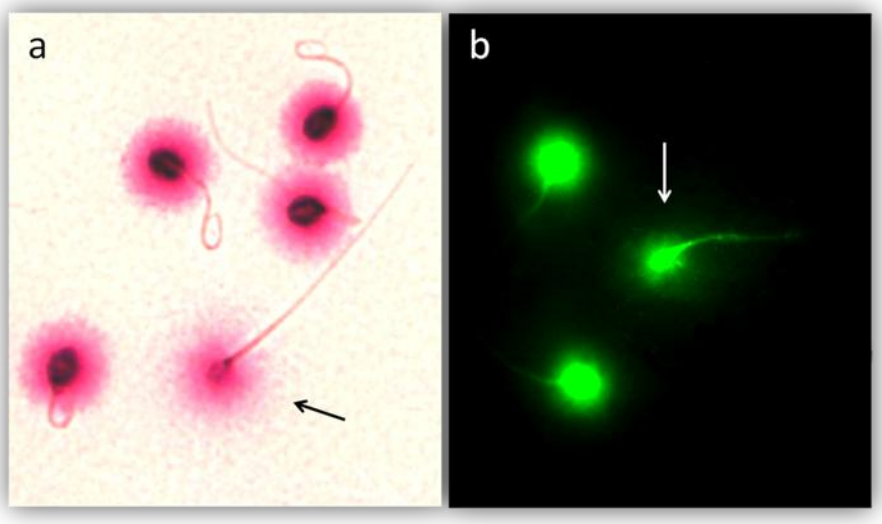


Figure 1. Sperm DNA fragmentation on canine sperm assessed with the sperm chromatin dispersion test and visualized with bright-field (a) or fluorescence microscopy (b). The arrows indicate the spermatozoa with fragmented DNA.

Although both staining methods have been widely used to visualize sperm DNA damage in other species (Fernández et al. 2005, Enciso et al. 2006, García-Macías et al. 2007), the results obtained with each staining has not been compared yet.

Sperm DNA analysis has also been used to determine the success of certain assisted reproduction techniques, such as cryopreservation of semen. The evaluation of chromatin integrity of fresh, cooled and frozen-thawed canine sperm, has been assessed previously using the SCSA technique (Garcia-Macias et al. 2006, Prinosilova et al. 2012, Núñez-Martinez et al. 2005). Although, the SCDt has been previously used to analyze changes due to cryopreservation process in several animal species (Cortes-Gutierrez et al. 2008, Cortes-Gutierrez et al. 2009, López-Fernández et al. 2007, Lopez-Fernandez et al. 2008, Álvarez et al. 2008, Vernocchi et al. 2014, Alkmin et al. 2013); no studies have been performed in dogs for that purpose. Only some preliminary results have been published using fresh semen before this thesis (Hidalgo et al. 2010, Varesi et al. 2014).

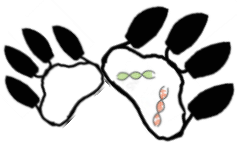
The quality of sperm doses used for artificial insemination, including the integrity of DNA, could be improved by selecting good quality spermatozoa with density gradient centrifugation (Phillips et al. 2012). Over the last years, a new method using single layer centrifugation (SLC) though a species-specific silane-coated silica colloid (Androcoll) has been developed at the Swedish University of Agricultural Sciences. Androcoll has been successfully used to select those motile morphologically normal spermatozoa with intact chromatin in different animal species (Dorado et al. 2013, Ortiz et al. 2014, Morrell et al. 2009). In this sense, Androcoll-C was developed as a specific SLC-colloid for dog sperm selection (Morrell et al. 2008); however, no

previous studies have been performed in dogs to evaluate the effect of SLC on sperm DNA fragmentation.

Regarding other sperm parameters, computer-assisted sperm morphometry analysis provides a number of objective parameters (Núñez-Martinez et al. 2005) which has been used to identify different subpopulations of spermatozoa within the mammalian ejaculate (Peña et al. 2005). In addition, sperm subpopulations analysis has become an issue of considerable interest for the evaluation of sperm samples (Núñez-Martinez et al. 2005, Saravia et al. 2007). A number of studies have attempted to address the role of sperm head morphometry on the prediction of freezability (Núñez-Martinez et al. 2007, Álvarez et al. 2008). Some of this studies have hypothesized that sperm head morphometric parameters may reflect chromatin organization (Núñez-Martinez et al. 2007, Álvarez et al. 2008, Lange-Consiglio et al. 2010), since subtle changes of sperm head can be related to abnormal DNA structure (Núñez-Martinez et al. 2007). In addition, the use of artificial insemination with shipped chilled dog semen has become very popular among breeders (Smith-Carr 2006). Some sperm parameters could be affected during cool storage which may affect sperm quality and fertility. In this sense, Hidalgo et al. (2015) has shown that sDFI starts to increase after 2 days of cool storage in canine semen samples. Most of these studies used SCSA to analyze the sperm DNA integrity, but until now, there were no studies that use the SCDt to evaluate the relationship between sperm morphometric subpopulation and sDFI on canine cool-storage sperm.

Cryopreservation of sperm collected from the epididymis allows the use of genetic material post-mortem or after orchiectomy from valuable animals or

endangered species (Thomassen and Farstad 2009). During the transit along the epididymis, spermatozoa suffer morphological and functional changes as part of its maturation process, which allows them to acquire progressive motility and fertilising capacity (Fouchecourt et al. 2000). Chromatin condensation begins during spermiogenesis, when the histones are first replaced by transition proteins and finally by small, basic protamines. These protamines form inter- and intramolecular disulfide bonds to fully condense chromatin. The site in the epididymis where the main condensation takes places in the dog is the caput-corpus transit, from this point on, the degree of DNA condensation is very similar to the ejaculate (Grimes Jr et al. 1997). In dogs, sperm DNA integrity has been evaluated in fresh (Núñez-Martinez et al. 2005, Lange-Consiglio et al. 2010, Urbano et al. 2013), cooled (Hidalgo et al. 2015) and frozen-thawed (Urbano et al. 2013) semen samples; however, there are few reports that assessed DNA integrity on epididymal canine sperm (Garcia-Macias et al. 2006, Varesi et al. 2014) and none of them evaluates DNA fragmentation dynamics. Moreover, SCSA and SCDt have been successfully used to evaluate canine sperm DNA fragmentation separately (Núñez-Martinez et al. 2005, Hidalgo et al. 2010, Prinosilova et al. 2012, Urbano et al. 2013, Varesi et al. 2014, Hidalgo et al. 2015,); but their results have not been compared yet.



OBJETIVOS

OBJECTIVES

OBJECTIVES

Several objectives are included in the present Doctoral Thesis as part of each publication:

OBJECTIVE 1 (First publication): *DNA integrity of canine spermatozoa during chill storage assessed by the sperm chromatin dispersion test using bright-field or fluorescence microscopy.* M Hidalgo, M Urbano, I Ortiz, S Demyda-Peyras , MR Murabito, MJ Gálvez, J Dorado (2015).

To evaluate the effect of chill storage for up to 96 hours on canine sperm DNA fragmentation assessed by the SCDt under bright-field or fluorescence microscopy.

OBJECTIVE 2 (Second publication): *Effect of cryopreservation and single layer centrifugation on canine sperm DNA fragmentation assessed by the sperm chromatin dispersion test.* M Urbano, J Dorado, I Ortiz, JM Morrell, S Demyda-Peyras, MJ Gálvez, L. Alcaraz, L Ramírez, M Hidalgo (2013).

To assess the effect of freezing and thawing on dog sperm DNA fragmentation using the SCDt and to determine whether or not the sperm selection by single layer centrifugation using Androcoll-C improves sperm DNA longevity in SLC-selected frozen-thawed dog semen samples.

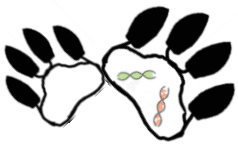
OBJECTIVE 3 (Third publication): *Identification of sperm morphometric subpopulations in cooled-stored canine sperm and its relation with sperm DNA integrity.* M Urbano, I Ortiz, J Dorado, M Hidalgo (2017).

To identify different morphometric subpopulations in cooled-stored canine sperm and their patterns of distribution during cool-storage and to determine whether or not morphometric sperm subpopulations are related to sperm DNA integrity assessed by the SCDt.

OBJECTIVE 4 (Fourth publication): *Comparison of DNA fragmentation of frozen-thawed epididymal canine sperm using SCSA and SCDt.* I Ortiz, M Urbano, J Dorado, JM Morrell, E Al-Essawe, A Johannisson, M Hidalgo.

To compare the sperm DNA fragmentation of frozen-thawed epididymal canine sperm using the SCSA and SCDt.

OBJECTIVES



OBJETIVOS

OBJETIVOS

Esta Tesis Doctoral incluye diversos objetivos específicos referidos a cada una de las publicaciones que la conforman:

OBJETIVO 1 (Primera publicación): *DNA integrity of canine spermatozoa during chill storage assessed by the sperm chromatin dispersion test using bright-field or fluorescence microscopy.* M Hidalgo, M Urbano, I Ortiz, S Demyda-Peyras , MR Murabito, MJ Gálvez, J Dorado (2015).

> Evaluar el efecto de la refrigeración sobre la fragmentación del ADN canino, durante 96 horas de refrigeración, usando el test de dispersión de la cromatina espermática mediante microscopía de campo claro y fluorescencia.

OBJETIVO 2 (Segunda publicación): *Effect of cryopreservation and single layer centrifugation on canine sperm DNA fragmentation assessed by the sperm chromatin dispersion test.* M Urbano, J Dorado, I Ortiz, JM Morrell, S Demyda-Peyras, MJ Gálvez, L. Alcaraz, L Ramírez, M Hidalgo (2013).

> Valorar el efecto de la congelación sobre la fragmentación del ADN canino mediante el test de dispersión de la cromatina espermática y determinar si la centrifugación coloidal de una sola capa usando Androcoll-C mejora la longevidad del ADN en las muestras seleccionadas y congeladas de semen de perro.

OBJETIVO 3 (Tercera publicación): *Identification of sperm morphometric subpopulations in cooled-stored canine sperm and its relation with sperm DNA integrity.* M Urbano, I Ortiz, J Dorado, M Hidalgo (2017).

Identificar diferentes subpoblaciones morfométricas así como su distribución en el esperma refrigerado de perro y determinar si esas subpoblaciones están relacionadas o no con la integridad del ADN mediante el test de dispersión de la cromatina espermática.

OBJETIVO 4 (Cuarta publicación): *Comparison of DNA fragmentation of frozen-thawed epididymal canine sperm using SCSA and SCDt.* M Urbano, I Ortiz, J Dorado, JM Morrell, E Al-Essawe, A Johannisson, M Hidalgo.

Comparar la fragmentación del ADN del esperma congelado de epidídimo de perro usando el SCSA y el SCDt.

CAPÍTULOS

CHAPTERS

CHAPTER 1

DNA integrity of canine spermatozoa during chill-storage assessed by the sperm chromatin dispersion test using bright field or fluorescence microscopy

M Hidalgo, M Urbano, I Ortiz, S Demyda-Peyras, MR Murabito, MJ Gálvez, J Dorado

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DNA integrity of canine spermatozoa during chill storage assessed by the sperm chromatin dispersion test using bright-field or fluorescence microscopy

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ABSTRACT

The objective of this study was to evaluate the effect of chill storage on canine sperm DNA fragmentation assessed by the sperm chromatin dispersion test using bright-field microscopy with Wright solution (sDF-B) or fluorescence microscopy with propidium iodide (sDF-F). The relationship and agreement between the results obtained with both staining methods were analyzed. The values of DNA fragmentation indexes (sDF-F and sDF-B) were compared at each time of chill storage (0, 24, 48, 72, and 96 hours). Additionally, the sperm DNA fragmentation rate (slope) was compared between the methods during chill storage. Good agreement and no significant differences between values obtained with both staining procedures were observed. Finally, the effect of chill storage for up to 96 hours was assessed on sperm motility parameters and DNA fragmentation indexes. Significant differences were found after 48 hours of chill storage, obtaining greater values of fragmented DNA. Progressive sperm motility was lower just after 96 hours of chill storage, and no effect was found in total sperm motility. In conclusion, the Sperm-Halomax kit, developed for canine semen and based on the sperm chromatin dispersion test, can be used accurately under bright-field or fluorescence microscopy to assess the sperm DNA integrity of canine semen during chill storage. The sperm DNA fragmentation index increased after 48 hours of chill storage, thereby detecting sperm damage earlier than other routine sperm parameters, such as sperm motility.

1. Introduction

Sperm quality has been traditionally evaluated using subjective parameters included in the spermiogram [1]. These results have been correlated with fertility; however, this relationship is not always as accurate as required. Consequently, developing and using more discriminatory *in vitro* tests to predict the fertilizing potential of a semen sample continues to be a priority [2]. Recently, the analysis of sperm DNA integrity has been proposed as an important index of fertility potential *in vitro* [3]. In humans, sperm DNA fragmentation has been used to explain infertility in semen samples with normal results in the spermiogram [4]. When assisted reproductive techniques, such as IVF or intracytoplasmic sperm injection, are used, the evaluation of sperm chromatin integrity is even more important [5].

Several techniques for sperm DNA assessment have been developed in the last years, including the sperm chromatin structure assay (SCSA), *in situ* nick translation, terminal deoxynucleotidyl transferase–mediated dUTP

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nick-end labeling, acridine orange test, or comet assay [6–8]. However, some of those methods are laborious and require expensive equipment or the use of purified enzymes for optimal analysis. This fact makes difficult its application in routine sperm quality analysis. To develop a new, accurate, easy, and quick technique for sperm DNA fragmentation assessment, the sperm chromatin dispersion test (SCDt) has been developed during the last few years in humans [7,9] and thereafter in others animal species [10–16]. Tests such as SCSA or SCDt require a denaturalization step to detect the DNA fragments or the potential breaks in the DNA [17]. When the SCDt is performed, sperm with fragmented DNA produce large and spotty halos of dispersed chromatin around the nuclear core (residual nucleus). Sperm nuclei with unfragmented DNA produce very small and compact halos of chromatin dispersion. Because of the relative size of the sperm halos, they may be easily visualized under bright-field or fluorescence microscopy. In spite of both methods having been used previously to evaluate sperm DNA fragmentation, the agreement of the results obtained has not been compared yet.

Sperm DNA can be affected by semen preservation procedures, such as sperm cooling [15,18]. Additionally, the stability of sperm DNA during long-term chill storage may not correspond with the results of routine semen parameters (i.e., sperm motility). Because of this, not only should good sperm motility or membrane integrity be preserved but also DNA integrity. In this sense, it would be useful to know the effect of chilling on canine sperm including other parameters, such as DNA fragmentation.

Previous studies have evaluated the sperm DNA fragmentation of cooled–stored semen using the SCDt in different animal species [19]; however, in dog semen samples, there is little information. There are some reports regarding the evaluation of chromatin integrity of fresh, chilled, and frozen–thawed dog sperm [12,20,21]; however, all of them have been performed using the SCSA. Few studies have been recently published using the SCDt to assess fresh and frozen–thawed epididymal or ejaculated dog sperm DNA integrity [22–24]. To our knowledge, no studies have been performed to evaluate canine chilled-stored semen samples using the SCDt, a simple and cost-effective method. Therefore, the objective of this study was to evaluate the effect of chill storage for up to 96 hours on canine sperm DNA fragmentation assessed by the SCDt under bright-field or fluorescence microscopy.

2. Materials and methods

2.1. Animals

Four healthy male dogs of different breeds (two Spanish Greyhounds, one German Shorthaired Pointer, and one crossbreed), aged from 4 to 5 years old, were used as semen donors. The dogs were housed in groups in indoor–outdoor runs with natural light, fed with commercial dry food, and received water *ad libitum*. All the dogs were trained for semen collection before the experiments. All animal procedures were performed in accordance with the Spanish laws for animal welfare and experimentation.

2.2. Semen collection

Semen samples were collected by digital manipulation in the presence of a teaser bitch. The second and third fractions of each ejaculate were collected into two, separate, calibrated, prewarmed plastic tubes connected to a plastic funnel (Canine Collection System; Minitüb, Tiefenbach, Germany). The four dogs were alternated for semen collection, selecting three dogs each time. Thereafter, the sperm-rich fractions of the three ejaculates were pooled on each occasion to increase semen volume and eliminate potential variability between donors. A total number of 18 ejaculates were collected and pooled in six sampling occasions. The ejaculates from the four dogs were equally collected and distributed through the pooled semen samples.

2.3. Semen processing

After collection, the volume of each pool of ejaculates was measured in a graduated tube (mL) and recorded. The sperm concentration was measured using a sperm photometer (Spermacue; Minitube). Sperm morphology was assessed on smears stained with Diff-Quick (Medion Diagnostics AG, Düdingen, Switzerland) under light microscopy using a × 40 objective (Olympus BH-2; Olympus Optical Co., Ltd., Tokyo, Japan). Sperm motility and DNA integrity were analyzed as described below. All the samples included in the study had at a minimum: morphologically normal sperm greater than 72%, sperm concentration greater than or equal to 430×10^6 sperm/mL, objective total motility greater than 89%, and progressive motility greater than 63%.

After evaluation, the remaining of each pooled semen sample was extended in a Tris-based commercial extender (Byladil Fraction A: 1210 g of Tris, 690 g of citric acid, 5 g of fructose, per 500 mL; Minitube) by adding 20% egg yolk (v:v). The diluted semen was placed into screw cap–closed sterile vials and conserved in the dark at room temperature (22 °C) for 10 minutes. Thereafter, semen samples were placed into a sperm container (Equitainer, Minitube) and allowed to cool gradually (0.3 °C/min) to 5 °C for up to 1 hour. Finally, the vials were put into a refrigerator and stored at 5 °C for 4 days. Semen samples were analyzed immediately after collection (fresh semen; T0) and after 24 (T24), 48 (T48), 72 (T72), and 96 hours (T96) of chill storage at 5 °C. For each analysis, a 100-μL aliquot was collected from fresh semen or from the 5 °C preserved semen samples, after gentle resuspension by agitation. The aliquots of fresh and chilled-stored semen samples were prewarmed into a 38 °C incubator for 10 minutes before the evaluation of sperm motility and DNA fragmentation.

2.4. Computer-assisted sperm motility analysis

Sperm motility was analyzed using the Sperm Class Analyzer (Microptic SL, Spain). Briefly, an aliquot of each pooled semen sample was diluted (25×10^6 sperm/mL) in Byladil A and incubated for 5 minutes at 38 °C. Then, a prewarmed Makler chamber (Sefi Medical Instruments

Ltd., Haifa, Israel) was loaded with 5 μL of sample, and a minimum of 200 sperm were counted under a phase-contrast microscope (Eclipse 50i; Nikon, Tokyo, Japan) with a prewarmed stage at 38 °C at × 100 magnification. At least three microscopic fields per drop were filmed at random. Total motility (%) was defined as the percentage of the sperm with mean average path velocity (VAP) greater than 15 μm/s, whereas objects with a VAP less than 10 μm/s were considered immobile. Progressive motility (%) was defined as the percentage of sperm with VAP greater than 50 μm/s and straightness ratio ([straight line velocity/VAP] × 100) greater than 75%.

2.5. Assessment of sperm DNA fragmentation

Sperm DNA fragmentation was assessed using the SCDt with the Sperm-Halomax commercial assay (Halotech DNA SL, Madrid, Spain), designed for canine semen, following the manufacturer's instructions.

2.5.1. Preparation of the semen samples

Semen samples were diluted in PBS (Sigma–Aldrich Chemie GmbH, Steinheim, Germany) to a final concentration of 5×10^6 sperm/mL. An aliquot of 25 μL was added to a vial containing 50-μL liquid low-melting agarose at 38 °C. This vial was previously immersed in a water bath at 90 °C to 100 °C for 5 minutes and subsequently left to warm for a further 5 minutes at 38 °C. Then, a drop of the sperm agar mixture was placed on a pretreated slide provided by the kit and covered with a 22 × 22-mm cover glass (Deltalab SL, Barcelona, Spain). The preparation was placed on a cooled metal plate and then stored in a refrigerator at 5 °C for 5 minutes. After that, the coverslip was carefully removed by sliding gently with the finger and not to leave it up directly. Thereafter, the slide was immersed (in a horizontal position) into a bath of 10 mL of lysis solution (containing β-mercaptoethanol) for 5 minutes at room temperature (22 °C). Subsequently, the preparation was maintained at room temperature and introduced into a bath of distilled water for 5 minutes and then immersed in three successive baths of ethanol at 70%, 90%, and 100% for 2 minutes each. Finally, the slides were allowed to air-dry before staining. Two slides were prepared for each semen sample and stained as follows.

2.5.2. Staining for bright-field microscopy evaluation

Slides were stained in Wright solution (Cat. no. 1.01383.0500; Merck) 1:1 in Phosphate Buffer (pH 6.88; Cat. no. 1.07294.1000; Merck) for 15 minutes keeping the slide in a horizontal position. After that, the dying solution was removed by draining off the excess of stain. Then, the slide was briefly and ligthly washed in tap water. Finally, the slides were air-dried and sealed using Eukitt mounting medium (Kindler & Co., Freiburg, Germany) and a coverslip. The slides were examined using light microscopy evaluation (Olympus BH-2, Olympus Optical Co., Ltd.).

2.5.3. Staining for fluorescence microscopy evaluation

The slides were stained with 5 μL of propidium iodide (P4170-10MG; Sigma–Aldrich Quimica, S.L., Madrid, Spain), mounted with 5 μL of antifading solution (Vectashield; Vector Laboratories, Inc., Burlingame, CA, USA), covered with a coverslip, and sealed with fingernail. The prepared slides were observed using a microscope (Olympus BX-40; Olympus U-RFL-T, Tokyo, Japan) outfitted with fluorescence using a U-ND25-2 filter (460–490 nm excitation filter).

2.5.4. Interpretation

Five hundred sperm were analyzed in each slide. Sperm with fragmented DNA showed a large halo of spotty halo of chromatin dispersion, and sperm with unfragmented DNA only showed a small and compact halo of chromatin dispersion (Fig. 1). The sperm DNA fragmentation index (sDFI) was calculated as the percentage of sperm with fragmented DNA over the total number of sperm counted under bright field (sDF-B, %) or fluorescence microscopy (sDF-F, %).

2.6. Experimental design and statistical analysis

Normality of the data distributions and variance homogeneity were evaluated using the Kolmogorov–Smirnov and Cochran tests, respectively. The results were expressed as the mean ± standard error of the mean. All statistical analyses were performed using SPSS v20.0 (IBM SPSS Statistics, Armonk, NY, USA) and GraphPad Prism v.6 (GraphPad Software, San Diego, CA, USA) for Mac OS X. The level of significance was set at $P < 0.05$.

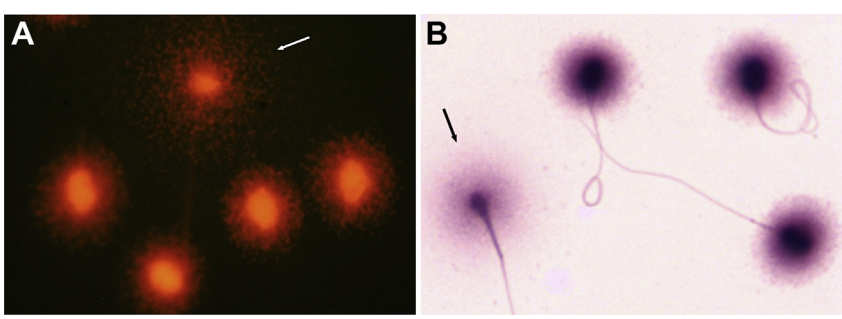

Fig. 1. Sperm DNA fragmentation of dog sperm after the sperm chromatin dispersion test using (A) fluorescence with propidium iodide and (B) bright-field microscopy with Wright staining. Sperm showing a large halo of chromatin dispersion containing fragmented DNA (arrows). Sperm nuclei that exhibited small and compact halos of chromatin dispersion corresponded to spermatozoa with unfragmented DNA.

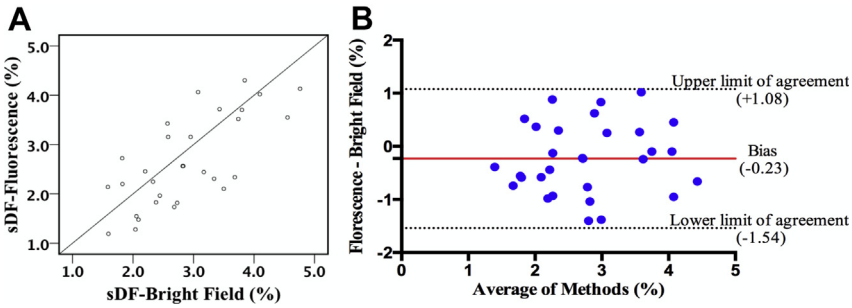

Fig. 2. Results of correlation and Bland–Altman analysis. (A) Scatter plot of correlation between sperm DNA fragmentation (sDF) values obtained with fluorescence and bright-field microscopy. Significant moderate correlation ($r = 0.72$; $P < 0.01$) was found between the methods. (B) Bland-Altman plot showing the agreement in the analysis of sperm DNA fragmentation between fluorescence and bright field microscopy. Each of the paired measures is represented by assigning the average of the two rates in abscissa and the difference between the two measurements in ordinate. The solid line represents the mean difference (bias), and the two dashed lines are the 95% limits of agreement (±1.96 standard deviation from the mean difference).

2.6.1. Experiment 1: Relationship, agreement, and reliability of sperm DNA fragmentation analysis using fluorescence or bright-field microscopy

The relationship and concordance between the results of sperm DNA fragmentation evaluated under fluorescence (sDF-F) or bright-field microscopy (sDF-B) were assessed across the entire set of semen samples analyzed. Correlation analysis was performed using the Pearson coefficient (r value). Scatter plots presented visual images of the data. The agreement and reliability between the results obtained with both methods were assessed using the Bland–Altman plot and the intraclass correlation coefficient (ICC). Comparisons between sDF-F and sDF-B at each time of cool storage (T0, T24, T48, T72, and T96) were performed using the Student t test. Finally, the rate of sperm DNA fragmentation per hour (slope) was compared between the staining methods. For that purpose, a linear regression analysis was performed and the intercepts and slopes of the regression equations of both methods (fluorescence and bright field) were compared by analysis of covariance using GrahPad Prism.

2.6.2. Experiment 2: Effect of chill storage for up to 96 hours on canine sperm motility and DNA integrity

The data of the sperm DNA analysis obtained with both methods were averaged into a single value: the sDFI (%). Then, the effect of storage time on sperm motility and sDFI was evaluated by ANOVA followed by the Duncan method.

3. Results

3.1. Experiment 1: relationship, agreement, and reliability of sperm DNA fragmentation analysis using fluorescence or bright-field microscopy

The correlation analysis between values of sperm DNA fragmentation obtained with each technique (fluorescence or bright field) showed significant moderate positive correlation between both methods ($r = 0.72$; $P < 0.01$, Fig. 2A). The Bland–Altman plot represented in Figure 2B included all the measures between the 95% limit of agreement, indicating good agreement between the staining methods. Additionally, the ICC obtained between the two staining techniques used was very high (ICC = 0.84), indicating good agreement between both methods.

The comparison of the sDFIs (sDF-F vs. sDF-B) at each time of chill storage (0, 24, 48, 72, and 96 hours) showed no significant differences between the methods (Fig. 3).

The velocity of sperm DNA fragmentation per hour is represented in Figure 4. No significant differences were found between the fluorescence versus bright-field techniques in the intercepts (1.84 vs. 2.05) and slopes (0.0170 vs. 0.0175) of both regression equations, respectively. This indicates that both staining methods give the same values of sperm DNA fragmentation rate during chill storage.

3.2. Experiment 2: Effect of chill storage on canine sperm motility and DNA integrity

Table 1 shows the results of averaged data of sperm DNA fragmentation (sDFI) assessed with both techniques during short-term chill storage (96 hours). Significant effect of sperm cooling was found, showing higher values of sDFI ($P < 0.001$) after 48 hours of chill storage. Progressive sperm motility was significantly lower ($P < 0.05$) after 96 hours of chill storage. No significant differences were found in total sperm motility during chill storage (Fig. 5).

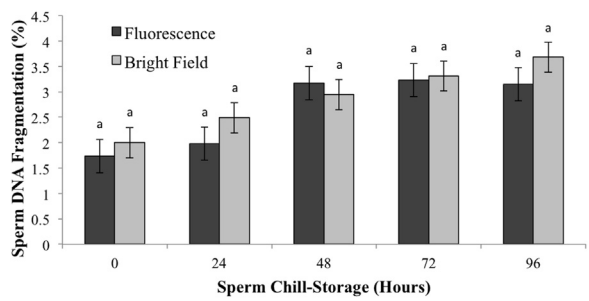

Fig. 3. Comparison of sperm DNA fragmentation between the methods at different times of chill storage. Values are expressed as the mean ± standard error of the mean. [a]No significant differences were found between methods ($P > 0.05$).

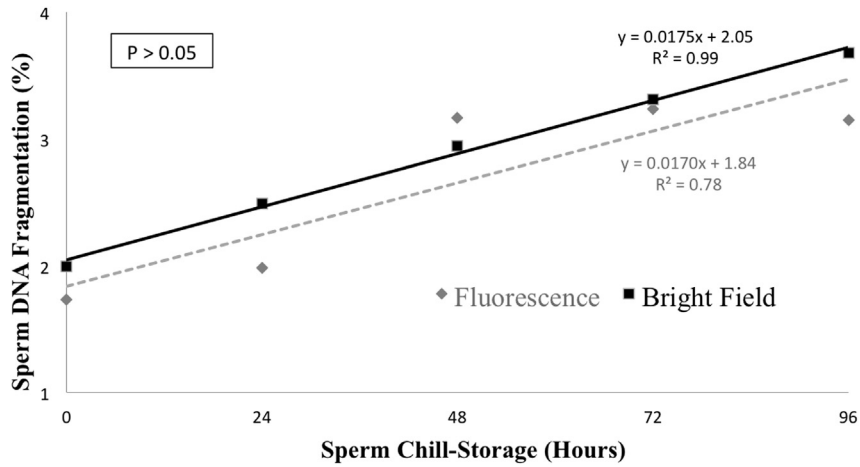

Fig. 4. Comparison of sperm DNA fragmentation per hour (slope) between the methods during chill storage. No significant differences were found between fluorescence versus bright-field techniques in the intercepts and slopes of both regression equations, respectively.

4. Discussion

In the present study, the sperm DNA integrity was assessed during chill storage using the Sperm-Halomax kit for canine semen on the basis of the SCDt. Two methods available for sperm DNA fragmentation analysis using the Sperm-Halomax kit were used: fluorescence (propidium iodide) and bright-field microscopy (Wright staining). Both techniques have been widely used to visualize sperm DNA damage in other species [9,11,25]. However, to our knowledge, this is the first attempt to compare the results obtained with each method.

For that purpose, we first analyzed the relationship between both methods. Our results showed a significant moderate correlation between the results of DNA fragmentation analyzed under fluorescence and bright-field microscopy. However, the correlation coefficient does not measure the agreement between methods but only the strength of their linear association [26]. A high correlation between methods is not always related to good agreement. Because of that, the agreement between both techniques was assessed using the Bland–Altman method. Differences between the values of DNA fragmentation obtained with fluorescence or bright-field microscopy were within the 95% confidence interval limits, indicating a good level of agreement between the two methods. Finally, reliability between the methods (degree in which a set of measurements or a measuring methods gives consistent results) was measured using the ICC on a scale from 0 to 1, where ICC of 1 represents perfect reliability and ICC of 0 indicates no reliability. Our results showed an ICC of 0.83 (ICC from 0.71–0.90 is considered a good level of concordance) indicating good reliability between the methods. Summarizing, the combined analysis of correlation, Bland–Altman analysis, and ICC informs there were not only moderate correlation but also good agreement and reliability between both methods in assessing sperm DNA fragmentation.

After that, the ability of both techniques to evaluate the sperm DNA integrity during different chill storage durations was assessed. No differences were found when values of sperm DNA fragmentation were compared between methods at different times of chill storage. In this sense, both techniques are useful to evaluate the sperm DNA integrity of chilled-stored canine semen. However, that comparison was performed in static fixed times of chill storage. It has been proposed that the sperm DNA integrity is not a static parameter because longevity of sperm DNA decreases progressively in time [17]. Previous studies have been performed to assess dynamics of sperm DNA fragmentation to compare the rate of sperm DNA fragmentation between different treatments [14,15] instead of comparing baseline values. Because of that, a dynamic study of sperm DNA fragmentation during chill storage was also performed to compare the information obtained from both methods. For that purpose, we performed a statistical analysis of regression equations to evaluate the rate of fragmentation per hour (slope) obtained with each staining procedure and then to assess possible variations between slopes attributable to the differences between methods. Our results showed no differences between staining's slopes; thus, we could say that

Table 1
Sperm DNA fragmentation index (sDFI) during chill storage.

Variable	Time of chill storage (hours)				
	T0	T24	T48	T72	T96
sDFI (%)	1.87 ± 0.06[a]	2.24 ± 0.21[a]	3.06 ± 0.28[b]	3.27 ± 0.33[b]	3.42 ± 0.35[b]

Values are expressed as mean ± standard error of the mean.
[a,b]Different letters indicate significant differences ($P < 0.001$).

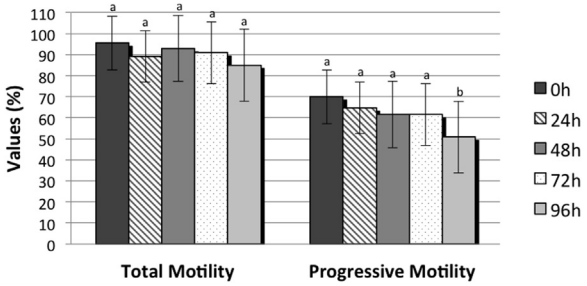

Fig. 5. Values of sperm motility parameters during chill storage (96 hours). Values are expressed as the mean ± standard error of the mean. [a,b]Different letters indicate significant differences ($P < 0.05$).

the information obtained in terms of rate of DNA fragmentation per hour is the same when chilled-stored canine semen is evaluated using fluorescence or bright-field microscopy. Consequently, both methods could be used accurately to evaluate the longevity of sperm DNA integrity of canine semen during chill storage.

Regarding the visual analysis of sperm with both techniques, we obtained a clear visualization of the halos of dispersed chromatin using both methods as we can see in Figure 1. This is particularly interesting when bright-field microscopy is used. It has been shown that using conventional bright-field microscopy after Wright staining, the sperm tails remain intact; thus, discrimination from other possible cell types can be easily accomplished [9,11]. On the contrary, fluorescence could be found preferable to bright field because of its brighter contrast; however, this subjective preference is just based on the visual appreciation of the technician and not in significant differences in the statistical analysis. Because no differences were found in the sDFIs between both methods, the assessment of sperm DNA integrity using bright-field microscopy offers an alternative method because the equipment required to perform this determination is quite less expensive. This also makes easier its use in routine analysis of canine semen.

On the other hand, given that no differences were found between fluorescence and bright-field microscopy analysis, in experiment 2, values of sperm DNA fragmentation obtained with both methods were pooled in a single index to evaluate the effect of chill storage for up to 96 hours on sperm DNA integrity, using sperm motility as a control parameter. The evaluation of routine sperm parameters, such as sperm motility, has been widely used to determine differences between cooling methods, including the effect of extenders [27–30], centrifugation protocols [31], seminal plasma [32], antioxidants [33,34], animal-free protein cryoprotectants [33], or storage time at different conditions [20]. However, in some cases, these kinds of analyses are not enough to determine the fertility of an individual [35]. For this reason, the evaluation of sperm chromatin structure has been proposed as a complementary test to determine male fertility potential [36].

There are some reports regarding the effect of cold storage on the sperm DNA integrity from other species using the SCDt [19], but little is known in dogs. The effect of chill storage in dog semen has been previously evaluated using the sperm chromatin structure assay [20], an expensive and laborious test that normally is available only in specialized research facilities [7]. To our knowledge, this is also the first attempt to evaluate the effect of short-term chill storage (up to 96 hours) in the DNA status (fragmented or unfragmented) of dog sperm using the SCDt.

According to our results, sperm DNA fragmentation increased significantly after 48 hours of chill storage. These results are in line with those reported by other authors using the SCDt for stallion semen, showing greater values of DNA fragmentation after 48 hours of cool storage [15]. Furthermore, in a different study, it was found that stallions classified as subfertile showed an increased susceptibility to denature or decline in chromatin quality between 20 and 31 hours of cold storage at 5 °C using the SCSA technique [18]. On the contrary, dog sperm chromatin integrity assessed with the SCSA technique did not change significantly during 10 days of chill storage using a commercial extender for dog semen chilling [20]. The aforementioned result could be due to the lysis solution used in the SCDt, which alters the chromatin in a different manner than the acid detergent used in SCSA, causing a disintegration of the protein scaffold, which may explain the differences between both techniques as described previously [10]. The question is whether or not the different methods available to measure DNA integrity of semen, particularly SCSA and SCDt, reveal the same type of damage and if the results are comparable for dog semen analysis [17]. Differences between these two methods could be due to the fact that chromatin condensation depends on the different nature of interaction between DNA and protamines and among protamines themselves [10]. The sperm chromatin structure assay method determines the extent of cellular DNA denaturation by measuring abnormalities in continuous double-stranded DNA using acridine orange staining to label the double-stranded DNA with green and the single-stranded DNA with red [8,17]. Sperm chromatin structure assay also provides the percentage of immature sperm within the semen sample [8]. The SCDt assesses DNA breaks after the lysis of chromatin proteins [10]. Additionally, the SCDt has shown the ability to identify both nuclear protein loss and sperm DNA fragmentation [37]. It has been proposed that SCDt and SCSA could measure different sperm traits about chromatin status and DNA, and because of the complexity of chromatin structure, more than one test could be needed [10]. Then, it would be reasonable that differences in dog DNA integrity during chill storage could be attributable to differences between methods for sperm DNA assessment. Then, according to our results, the use of SCDt could show additional information to SCSA about dog sperm DNA damage during dog chill storage. Future studies are needed to compare dog sperm DNA analysis using SCSA and SCDt.

In the present study, as mentioned previously, sperm motility was evaluated as a routine control parameter to assess the success of dog sperm chill storage in comparison to the results obtained with DNA analysis. According to our results, no differences were found in total sperm motility during chill storage; only progressive sperm motility was lower after 96 hours of chill storage. This is in agreement with previous studies where long-term chill storage of dog semen did not change the motility of chilled dog semen up

to 96 hours [29,38]. However, in the present study, we observed an increment of sperm DNA fragmentation just after 48 hours of chill storage. It could suggest that chilled sperm shows DNA damage before the decline in sperm quality. Then, if routine parameters are used alone to evaluate the sperm quality of chilled-stored canine semen, we could use semen samples with good motility but low DNA integrity, which could result in lower fertility after artificial insemination. Therefore, the analysis of sperm DNA fragmentation could be an important tool to obtain supplementary information about the quality of chilled-stored dog semen.

In conclusion, Sperm-Halomax, developed for canine semen and based on the SCDt, can be used to assess the sperm DNA integrity of chilled-stored canine semen using both bright-field and fluorescence microscopy. The sDFI increased after 48 hours of chill storage, which could detect sperm damage earlier than other routine sperm parameters, such as sperm motility.

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CHAPTER 2

Effect of cryopreservation and single layer centrifugation on canine sperm DNA fragmentation assessed by the sperm chromatin dispersion test

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Effect of cryopreservation and single layer centrifugation on canine sperm DNA fragmentation assessed by the sperm chromatin dispersion test

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ABSTRACT

The aims of this study were: 1) to assess the effect of freezing and thawing on dog sperm DNA fragmentation index (sDFI) using the sperm chromatin dispersion test (SCDt); and 2) to determine whether or not the sperm selection by single layer centrifugation (SLC) using Androcoll-C improves sperm DNA longevity in SLC-selected frozen-thawed dog semen samples. Semen samples were collected from 4 dogs using digital manipulation. After collection, ejaculates were pooled and cryopreserved following a standard protocol. Sperm motility and morphology were assessed before freezing and after thawing as a control for the cryopreservation method used. In experiment 1, sDFI was analyzed immediately before freezing and after thawing (baseline values), showing no significant differences between fresh and frozen-thawed semen samples. In experiment 2, frozen-thawed semen samples were processed or not by SLC using Androcoll-C and longevity of DNA were assessed in terms of sDFI after 24 h of in vitro incubation at physiological temperature (38 °C). The results showed low values of sDFI in SLC-selected semen in comparison to unselected samples. In conclusion, no effect of cryopreservation was observed on baseline values of dog sperm DNA fragmentation. Additionally, SLC-selection using Androcoll-C improved longevity of frozen-thawed sperm DNA assessed by the SCDt.

1. Introduction

The reduction of fertilizing ability of semen after freezing and thawing has been associated with a reduced rate of sperm motility, normal sperm morphology and damage of the sperm membranes (Cortes-Gutierrez et al., 2008). Although preservation of both sperm membrane functions and motility are important parameters to determine sperm quality, they do not reveal changes associated with sperm DNA integrity, which has also been shown to be a useful parameter to evaluate the sperm quality (Aravindan et al., 1997). This is particularly interesting because DNA fragmentation correlates poorly with classical parameters of semen quality analysis. In this sense, subfertile men with an apparently normal spermiogram showed higher sperm DNA fragmentation index (sDFI), explaining the problem of infertility (Giwercman et al., 2003). Thus, sDFI should be considered an important parameter to assess together with the classic spermiogram.

Different techniques such as TUNEL (Terminal dUTP Nick-End Labeling), SCSA (Sperm Chromatin Structure

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Assay) or comet assay are commonly used to evaluate sperm DNA integrity. However, sometimes the methodology of these techniques is complicated or specific equipment such as a flow cytometer is required. An alternative method that provides reliable results with a simple methodology is the recently developed Sperm Chromatin Dispersion test (SCDt) (Fernández et al., 2003). SCDt is a simple microgel slide-based assay that has been used to assess sperm DNA fragmentation in a wide range of species. The SCDt was originally developed for humans (Fernandez et al., 2005). This method involves treating spermatozoa with an acidic solution to denature DNA prior to a lysing procedure that removes proteins and membranes. Thereafter, different research has been focused on modifying and adapting the SCDt to the unique chromatin structure of a range of domestic and wild animals, including bulls (Martínez-Pastor et al., 2009), rams (López-Fernández et al., 2008), bears (Álvarez et al., 2008) and donkeys (Cortes-Gutierrez et al., 2008). In dogs, some preliminary results have been published using fresh semen (Hidalgo et al., 2010).

The SCDt is commercialized for different animal species with the commercial kit Sperm Halomax® (Halotech DNA SL, Madrid, Spain). This is a quick and easy method based on the analysis of DNA fragmentation by visual fluorescence microscopic observation of sperm with a halo of chromatin dispersion around the nuclear core of the spermatozoa. Using this technique, spermatozoa with a large halo of dispersed chromatin surrounding a variable-sized nuclear core, correspond to those containing fragmented DNA.

Sperm DNA analysis has also been used to determine the success of certain assisted reproduction techniques, including cryopreservation of semen. In this way, the effect of freezing and thawing on canine sperm DNA integrity using the SCSA method has been shown previously (Prinosilova et al., 2012). The SCDt has been previously used to analyze changes due to cryopreservation in several animal species (Cortes-Gutierrez et al., 2008; Cortes-Gutierrez et al., 2009); however, to our knowledge, no studies have been performed in dogs for this purpose.

The sperm DNA fragmentation (sDF) using the SCDt can be analyzed by static or dynamic studies. In static studies, sDF is evaluated at the initial time, for example immediately after semen collection or after freezing and thawing (Cortes-Gutierrez et al., 2009). In dynamic studies, spermatozoa are subjected to a stressor and variations of sDF are measured over time. For example, dynamic studies have been performed in donkey and stallion semen incubated at 37 °C for 24 h, showing individual differences in the increasing of the baseline values of sDF over time (Cortes-Gutierrez et al., 2008). The increase of baseline values of sDF has been associated with sperm preparation for artificial reproduction techniques (Lopez-Fernandez et al., 2007) and subsequently with a reduction in fertility rates. However, despite all of these studies, most authors indicated that experience with the SCDt should be expanded (Cortes-Gutierrez et al., 2008).

On the other hand, the quality of sperm doses used for AI, including the integrity of DNA, could be improved, for example, by selecting "good quality" spermatozoa with density gradient centrifugation (DGC) (Phillips et al., 2012). Recently, a new method using single layer centrifugation (SLC) through a species-specific silane-coated silica colloid (Androcoll, patent applied for) has been developed at the Swedish University of Agricultural Sciences. Androcoll has been successfully used to select those motile morphologically normal spermatozoa with intact chromatin in different animal species (Morrell et al., 2005, 2009a; Thys et al., 2009; Chatdarong et al., 2010; Martinez-Alborcia et al., 2012). For example, SLC using Androcoll-E in horses improved the sperm quality of frozen-thawed semen samples (Morrell et al., 2009a) as well as the sperm quality of subfertile stallions (Morrell et al., 2011). Several studies have been performed in dogs to improve the sperm motility and viability of post-thaw canine sperm using DGC media formulated for use in humans (Dorado et al., 2011a, 2011d). Recently, Androcoll-C, a new specific SLC-colloid has been developed for its use in dogs (Morrell et al., 2008); however, to our knowledge, no previous studies has been performed in dogs to evaluate the effect of SLC on sperm DNA fragmentation.

The objectives of this study were: 1) to assess the effect of freezing and thawing on dog sperm DNA fragmentation using the SCDt; 2) to determine whether or not the sperm selection by single layer centrifugation using Androcoll-C improves sperm DNA longevity in SLC-selected frozen-thawed dog semen samples.

2. Materials and methods

2.1. Semen collection

Semen was obtained by digital manipulation from 4 healthy beagle dogs, aged 4–5 years. Three different dogs were collected on each occasion and thereafter, the sperm rich fraction of the three ejaculates was pooled. A total number of twelve ejaculates were collected and pooled in four sampling occasions. The ejaculates from the four dogs were equally collected and distributed through the pooled semen samples.

2.2. Semen evaluation

Semen volume (ml) was measured in a calibrated tube. Sperm concentration was determined using a sperm photometer (SpermaCue, Minitüb, Tiefenbach, Germany).

2.2.1. Computer-assisted sperm motility analysis (CASA)

Sperm motility was evaluated by CASA using the Sperm Class Analyzer (SCA V.5.0.1, Microptic SL, Barcelona, Spain), as described previously (Dorado et al., 2011e). The following sperm parameters were recorded: total motility (TM, %), progressive motility (PM, %), curvilinear velocity (VCL, μm/s), linear velocity (VSL, μm/s), average mean velocity (VAP, μm/s), linear coefficient (LIN, VSL/VCL × 100), straightness coefficient (STR, VSL/VAP × 100), wobble coefficient (WOB, VAP/VCL × 100), amplitude of lateral head displacement (ALH, μm) and beat cross frequency (BCF, Hz).

2.2.2. Sperm morphology

Sperm morphology was evaluated on slides stained with Diff-Quick® (Hidalgo et al., 2006). At least 200 spermatozoa were observed and the percentage of spermatozoa with normal morphology recorded.

2.2.3. Sperm DNA fragmentation analysis

The sperm DNA fragmentation analysis was performed using the sperm chromatin dispersion test (SCDt). For that purpose, the commercial kit Sperm-Halomax® (Halotech DNA SL, Madrid, Spain) developed for canine semen was used following the manufacturer's instructions.

In brief, one aliquot of each semen sample was diluted in phosphate buffer solution (PBS, Sigma-Aldrich Chemie GmbH, Steinheim, Germany) to a concentration of 5×10^6 sperm/ml. After 10 min of incubation at 38 °C, 25 μl of the dilution were added to a vial containing agarose gel. This vial was previously immersed in a water bath at 90–100 °C for 5 min and subsequently was left to warm for a further 5 min at 38 °C. Then, a drop of the sperm agar mixture was placed on the surface of each pre-treated slide provided in the kit and covered with a 22 × 22 mm coverslip. The preparation was placed on a metal plate, previously cooled, and then stored in a refrigerator at 5 °C for 5 min. After that, the coverslip was removed and the slide was immersed into a bath of 10 ml of lysis solution for 5 min. Subsequently, the preparation was introduced into a bath of distilled water for 5 min and then immersed in three successive baths of ethanol at 70%, 90% and 100% for 2 min each. Finally, the slides were allowed to air-dry before staining.

All the slides were stained using a commercial kit for green fluorescence staining (Halotech DNA SL, Madrid, Spain). The staining method allowed differentiation of fragmented and unfragmented sperm DNA according to the halo of chromatin dispersion around the sperm nuclei. Sperm showing a small and compacted halo of chromatin dispersion around a compacted nuclear core contained intact DNA (Fig. 1c). Sperm that displayed a large and spotty halo of chromatin dispersion around the nuclear core corresponded to those sperm with fragmented DNA (Fig. 1b). The sperm DNA fragmentation index (sDFI) was then calculated as the percentage of spermatozoa with fragmented DNA over the total number of sperm counted per slide (500 spermatozoa per sample).

Digital images were captured using a camera Olympus Altra 20 (Soft Imaging Solutions GmbH) mounted on a fluorescence microscopy (Olympus BX-40, Olympus U-RFL-T, Tokyo, Japan). To improve digital image acquisition and to discriminate sperm head/tail, some slides were counterstained with 1 μl of propidium iodide (P4170-10MG, Sigma–Aldrich Quimica, S.L. Madrid), before image acquisition. When double fluorescence staining (red emission for DNA and green emission for proteins) was used, the flagellum was clearly discriminated from the chromatin. The nuclear core of unfragmented sperm, which partially resisted protein depletion, exhibited a compacted red fluorescence emission with a yellow color in the middle where red and green fluorescence signals merged (Fig. 1a). Large halos of dispersed chromatin consisted primarily of fragmented DNA showing red fluorescence emission (Fig. 1a; arrow). Image analysis was performed using the analysis getIT software (Soft Imaging DT5). Images were modified using adobe Photoshop CS, version 8.0.1.

2.3. Sperm cryopreservation

Semen was frozen in two steps using a standard procedure for canine semen (CaniPRO™ Freeze, Minitüb, Tiefenbach, Germany) with the following modifications. In brief, after collection, each pool of semen was diluted 1:1 (v/v) with a culture medium for canine semen (CaniPRO™ APX2 AI, Minitüb, Tiefenbach, Germany) and then centrifuged at 700 × *g* for 10 min (22 °C). The sperm pellet was resuspended with CaniPRO™ Freeze A plus 20% egg yolk to a final concentration of 200×10^6 sperm/ml and it was slowly cooled to 5 °C within 1.5 h. After that, the extended semen was diluted to a final concentration of 100×10^6 sperm/ml in CaniPRO™ Freeze B (containing glycerol) plus 20% egg yolk and was stored 30 min at 5 °C. Finally, semen samples were loaded into 0.5 ml plastic straws at 5 °C, frozen horizontally in ranks placed 4 cm above the surface of liquid nitrogen (LN_2) for 10 min and stored into LN_2 tanks. Straws were thawed for sperm analyses in a water bath at 37 °C for 30 s, after a minimum period of storage of seven days.

2.4. Single layer centrifugation using Androcoll-C

Sperm selection was performed on frozen-thawed dog semen by single layer centrifugation (SLC) using Androcoll-C (Swedish University of Agricultural Sciences, Uppsala, Sweden). Androcoll-C consisted of a silane-coated silica colloid specifically formulated for canine semen (Morrell et al., 2008). For this purpose, 1 ml of Androcoll-C was pipetted into a 15 ml centrifuge tube. Then 0.8 ml of each frozen-thawed semen sample containing 100×10^6 sperm/ml was layered on top of the gradient before centrifugation (300 × *g*/20 min at 22 °C). Thereafter, the sperm pellet was diluted with 1 ml of CaniPRO™ ApX2 AI (Minitüb, Tiefenbach, Germany) to obtain a concentration of 25×10^6 sperm/ml and was evaluated as described previously. The sperm recovery rate was assessed using the SCA system to evaluate sperm concentration after SLC by the following formula:

$$\text{Sperm yield}\,(\%) = \left(\frac{\text{number of spermatozoa in the sperm pellet}}{\text{number of spermatozoa in the initial load}}\right) \times 100$$

2.5. Experimental design

All the experiments performed were replicated four times.

2.5.1. Experiment 1. Effect of cryopreservation on dog sperm DNA fragmentation

Sperm DNA analysis using the SCDt was performed in fresh and frozen-thawed semen samples at the initial time (T0), immediately after collection or after thawing respectively. Baseline values of sDFI were compared between fresh and frozen-thawed semen samples. Sperm motility and morphology were also compared before freezing and after thawing as control of the cryopreservation method used.

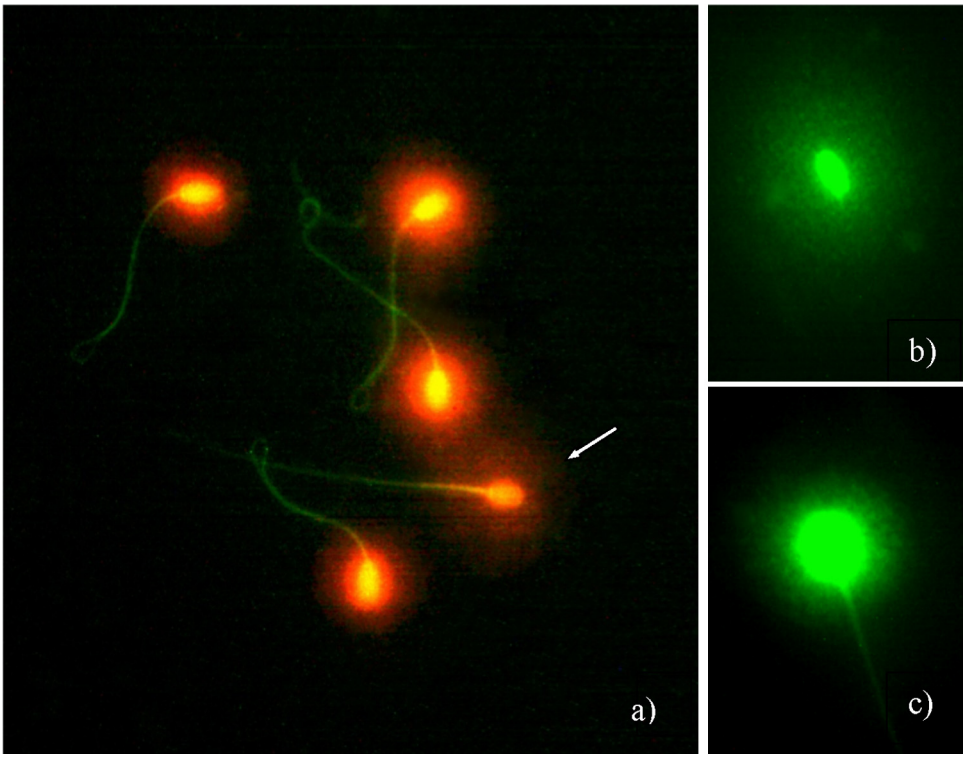

Fig. 1. Sperm DNA fragmentation in dog after SCDt. (a) SCD-processed sample with dual fluorescence; sperm nuclei with fragmented DNA exhibit a large and spotty halo of chromatin dispersion (arrow). In contrast, sperm nuclei that exhibited small and compact halos of chromatin dispersion corresponded to spermatozoa with unfragmented DNA. (b and c) SCD-processed sample with green fluorescence; DNA-fragmented spermatozoa (b) and DNA-unfragmented spermatozoa (c).

2.5.2. Experiment 2. Effect of SLC with Androcoll-C on sperm DNA longevity of frozen-thawed dog semen

This experiment was performed in order to determine if the sperm selection using Androcoll-C improves the sperm DNA longevity of frozen-thawed dog semen assessed with the SCDt. For this purpose, semen samples were divided in two aliquots after thawing: one of them was centrifuged with Androcoll-C (SLC-selected) according to the methodology described above and then incubated for 24 h at 38 °C. The other aliquot was used as control (unselected) and was incubated at the same conditions immediately after thawing. After incubation, sperm DNA fragmentation was assessed and compared between both SLC-selected and unselected frozen-thawed semen samples.

2.6. Statistical analysis

Normality of the data distributions and variance homogeneity were checked by the Kolmogorov–Smirnov and Cochran tests, respectively. Sperm motility, morphology and sDFI were compared between treatments by ANOVA. Sperm kinetic parameters were compared using the Kruskal–Wallis nonparametric test, followed by the Mann–Whitney *U*-test. The results were expressed as mean ± SEM. Correlations between sDFI and total sperm motility, progressive motility and normal sperm morphology was performed using the Pearson coefficient (*r* value). Scatter plots presented visual image of data. All analyses were performed with SPSS statistic package v15.0 (SPSS Institute Inc. Headquarters, Chicago, IL, USA). The level of significance was set at $P < 0.05$.

3. Results

3.1. Experiment 1. Effect of cryopreservation on dog sperm DNA fragmentation

Dog spermatozoa exhibited 2 different morphotypes of fragmented nucleus after SCDt. Morphotype 1 (MP-1) showed a small halo of chromatin dispersion and corresponds to unfragmented spermatozoa. Morphotype 2 (MP-2) showed a large halo of chromatin dispersion around the core and corresponds to fragmented spermatozoa.

Total sperm motility ($P < 0.05$) and progressive sperm motility ($P < 0.01$) were significantly higher in fresh semen versus frozen-thawed samples; however, no significant differences ($P > 0.05$) on sperm DNA fragmentation index were found between fresh and frozen-thawed semen ($1.41 \pm 0.07\%$ vs. $1.46 \pm 0.08\%$) when evaluated at the initial time (T0). No significant differences were found for normal sperm morphology between fresh and frozen-thawed semen samples (Table 1).

In contrast, sperm kinetic parameters were significantly ($P < 0.001$) higher in frozen-thawed semen except for amplitude of lateral head displacement (ALH), which was significantly higher in fresh semen. Curvilinear velocity

Table 1
Comparison of sperm motility, morphology and DNA fragmentation index (sDFI) between fresh semen and frozen-thawed semen samples immediately after thawing (T0).

Semen sample	Sperm parameters			
	Total motility (%)	Progressive motility (%)	Normal sperm morphology (%)	sDFI (%)
Fresh semen	94.25 ± 1.77	74.97 ± 2.99	84.62 ± 2.50	1.41 ± 0.07
Frozen-thawed	67.95 ± 7.01	48.40 ± 5.70	79.27 ± 3.72	1.46 ± 0.08
Statics	$P < 0.05$	$P < 0.01$	ns	ns

Values are expressed as mean ± SEM.
ns: no significant differences between treatments.

(VCL) was also higher in fresh semen but not significantly (Fig. 2).

3.2. Experiment 2. Effect of SLC with Androcoll-C on sperm DNA longevity of frozen-thawed dog semen

According to the results showed in Table 2, no motile sperm were observed in both unselected and SLC-selected frozen-thawed semen samples after 24 h of incubation at 38 °C. However, the percentage of sperm with normal forms was significantly higher ($P < 0.05$) in SLC-selected samples. Additionally, the sperm DNA fragmentation index was significantly lower ($P < 0.001$) in SLC-selected frozen-thawed semen samples.

The relationship between sperm motility, morphology and sperm DNA fragmentation index (sDFI) was assessed across all treatments (Fig. 3). No correlation between sDFI and total sperm motility was found ($r = -0.13$; $P = 0.63$; Fig. 3a). In addition, progressive sperm motility and sDFI were not correlated ($r = -0.12$; $P = 0.65$; Fig. 3b). However, the percentage of normal sperm morphology was significantly and negatively correlated with percentage of sperm with DNA fragmented ($r = -0.74$; $P = 0.001$; Fig. 3c).

4. Discussion

Traditionally, sperm motility and morphology have been used as control parameters to assess cryopreservation success (Linde-Forsberg, 1995). According to our results, both parameters were lower in cryopreserved semen samples than in fresh semen, as expected. However an unexpected result was found. Most of the sperm kinetic parameters were significantly higher in post-thaw semen samples. One possible explanation of this fact is the induction of sperm capacitation due to cryopreservation procedure. This issue has been previously described in wild boars (Flores et al., 2009). As a consequence, a temporal increase on post-thaw sperm velocity could be

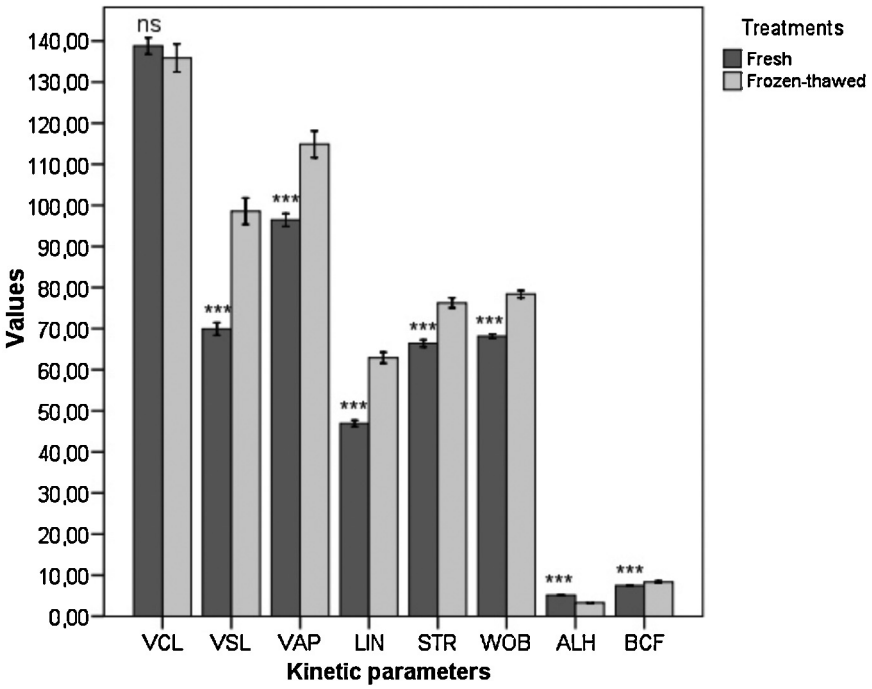

Fig. 2. Comparison of sperm kinetic parameters between fresh and frozen-thawed semen samples immediately after thawing (T0). (VCL) curvilinear velocity; (VSL) linear velocity; (VAP), average mean velocity; (LIN) linear coefficient; (STR) straightness coefficient; (WOB) wobble coefficient; (ALH) amplitude of lateral head displacement and (BCF) beat cross frequency. (ns) no significant differences between treatments. ***Significant differences between treatments ($P < 0.001$)

Table 2
Effect of single layer centrifugation (SLC) using Androcoll-C on sperm motility, morphology and DNA fragmentation index (sDFI) in frozen-thawed semen samples after 24 h incubation at 38 °C.

	Post-thawed sperm parameters			
	Total motility (%)	Progressive motility (%)	Normal sperm morphology (%)	sDFI (%)
Unselected	0.00	0.00	70.52 ± 5.44	2.73 ± 0.18
SLC-selected	0.00	0.00	86.91 ± 2.29	0.58 ± 0.00
Statics	ns	ns	$P < 0.05$	$P < 0.001$

Values are expressed as mean ± SEM.
ns: no significant differences between treatments.

due to the hyper-motility induced by sperm capacitation in frozen-thawed semen samples. However, Flores et al. (2008) showed that boar and donkey sperm responded very differently in their mean motion characteristics to freezing and thawing procedure. Due to this, the specific mechanism by which these modifications are produced maybe related to other factors, such as the osmotic stress produced by cryopreservation procedures (i.e. changes in mitochondrial activity, production of reactive oxygen species, etc.).

In the first experiment, we assessed the effect of cryopreservation on dog sperm DNA fragmentation. Using this technique in other animals, spermatozoa with fragmented DNA presented a big halo of chromatin dispersion around the core. In humans, the interpretation is different and includes 4 different morphotypes according to DNA migration (Fernández et al., 2003). According to our results, when SCDt was applied to dog spermatozoa, we identified 2 different morphotypes of fragmented nucleus. MP-1 corresponds to unfragmented DNA sperm and mainly contains DNA sequences that are highly sensitive to gentle alkaline environments. It is due to the presence of unpaired single-stranded DNA motifs or the presence of apurinic or apyrimidinic sites that are part of constitutive structural DNA modification (Zee et al., 2009). Morphotype 2 represented DNA fragmented sperm and is proposed as representing "true" DNA fragmentation, where both single- and double-strand breaks are present. This interpretation has been previously demonstrated in other animal species (Zee et al., 2009).

When sperm DNA fragmentation was compared between fresh and frozen thawed dog semen samples, our results showed that baseline values of sDFI were not affected by cryopreservation. There are previous reports, which showed different results about the effect of cryopreservation on sperm DNA integrity immediately after thawing. On the one hand, these results are in disagreement with those obtained by Khalifa et al. (2008), who ascertained that cryopreservation of bull semen induced a pronounced decrease in sperm chromatin stability immediately after thawing. In this direction, Fraser and Strzezek (2007) using the Comet assay, confirmed that freezing-thawing facilitates destabilization of boar sperm chromatin structures, making the spermatozoa more vulnerable to DNA fragmentation. Moreover, in contrast to all this, Koderle et al. (2009) detected even lower sperm DNA fragmentation values after thawing than in dog fresh semen. On the other hand, Prinosilova et al. (2012) using the Sperm Chromatin Structure Assay (SCSA) showed that cryopreservation did not cause any significant changes in dog sperm chromatin integrity immediately after thawing. Our results are in agreement with this study as well as with other previous reports performed in different animal species, such as rams (Peris et al., 2004), stallions (Lopez-Fernandez et al., 2007; Cortes-Gutierrez et al., 2009), donkeys (Cortes-Gutierrez et al., 2008) or humans (Donnelly et al., 2001; Isachenko et al., 2004). According to this, DNA damage is not a direct effect of freezing in dog semen. Thus, the stability of DNA molecule after thawing is likely to be an indirect consequence of the stressors associated with changes in temperature, osmolarity, etc. (Cortes-Gutierrez et al., 2008).

To investigate this theory further, we performed the second experiment. Therefore, cryopreserved semen was incubated in vitro at body temperature for 24 h, to replicate the viability of frozen-thawed dog sperm into the female reproductive tract of the bitch (approximately 12–24 h after AI). Although this is not comparable to the interaction that occurs between the spermatozoa and the uterus of

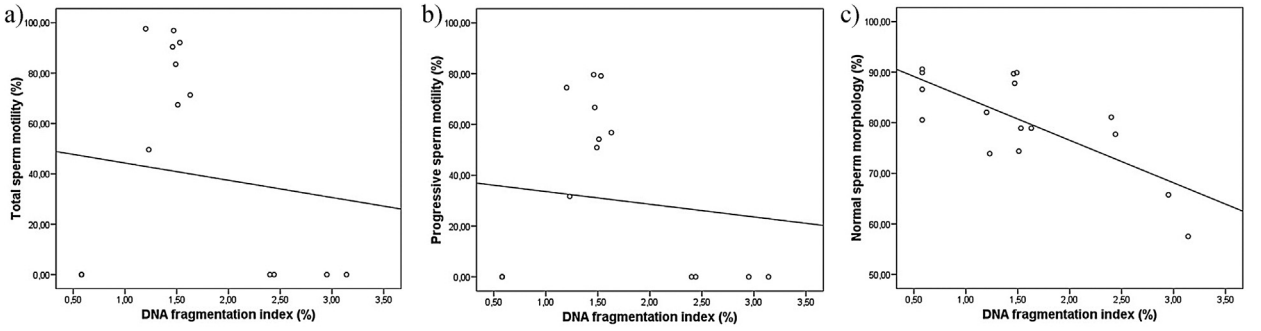

Fig. 3. The three plots illustrate the relationship between the percentage of sperm DNA fragmentation with total sperm motility (a), progressive sperm motility (b) and normal sperm morphology (c), respectively. Each value for all the sperm parameter shown was calculated across all treatments.

the bitch, it could help to determine strength and viability of frozen sperm in terms of DNA fragmentation. Additionally, it was suggested that a more accurate evaluation of the extent of the nuclear DNA fragmentation could be measured after incubating the sperm at biological temperature due to the level of sperm damage is much more intense (López-Fernández et al., 2007). In this sense Cortes-Gutierrez et al. (2008) showed that donkey sperm was more sensitive to DNA breakage when incubated at 37 °C in comparison to 25 °C or 4 °C.

Single layer centrifugation (SLC) using a silane-coated silica colloid has been previously used to select "good quality" spermatozoa from different species, including stallions (Cortes-Gutierrez et al., 2008) bulls (Macías García et al., 2009; Morrell et al., 2009a, 2009b) or dogs (Samardzija et al., 2006). According to our results, sDFI in frozen-thawed SLC-selected dog semen using Androcoll-C was lower than unselected samples, after 24 h of incubation at 38 °C. This results are in agreement with those observed in stallions where rate of DNA fragmentation after 24 h incubation at 37 °C was lower in semen samples subjected to colloid centrifugation (Dorado et al., 2011b, 2011c). Additionally, these authors found no differences in basal sDFI between SLC-selected and unselected semen. These results support the fact that most of the sperm DNA damage occurs during incubation, rather than in the first minutes after thawing (Crespo et al., 2013). Thus, assessing sperm DNA fragmentation immediately after thawing may show "only the tip of the iceberg" as hypothesized previously (Alkmin et al., 2013). However, when cryopreserved dog sperm were submitted to a stressor, sDFI of unselected samples increased and this increment probably depends on the initial damage of the DNA. This could explain the results obtained in the second experiment, where the combination of SLC-selection and incubation after thawing resulted in low values of sDFI.

Additionally, in frozen-thawed selected and unselected dog semen samples, no motile sperm were found after incubation. These results are similar to those obtained by Alkmin et al. (2013) in boars where SCDt was performed over time and motility was lower than 10% after 24 h incubation at 37 °C. As described above, sperm motility has been traditionally considered as the main criteria to ensure that the freezing technique was successful. However, sDFI values of SLC-selected frozen-thawed semen were lower than those obtained in unselected semen in spite of both samples showing no motile sperm. In addition, sDFI of SLC-selected samples was even lower than baseline values of sDFI in fresh semen, where sperm motility is highest. Moreover, according to our results, no relationship was found between sperm motility and sperm DNA fragmentation. Thus, there is no direct correlation between sperm motility and sperm DNA fragmentation in terms of sperm DNA longevity. This fact has already been demonstrated in humans, where subfertility in males with normal spermiogram was diagnosed based on a higher rate of sperm DNA fragmentation (Giwercman et al., 2003). Our results suggested a negative high correlation between sperm morphology and sperm DNA fragmentation. Similar results have been obtained recently in a retrospective analysis of sperm morphology and chromatin stability, assessed by SCSA, and their relation to fertility after AI (Nagy et al., 2013). In the latter study, fertility was significantly correlated only with the percentage of morphologically normal spermatozoa, a variable that negatively correlated with the percentage of DNA fragmentation at the time of SCSA, thus confirming the value of always having high numbers of morphologically normal spermatozoa in AI-doses. According to these results, sDFI could be an additional parameter for routine analysis of post-thaw sperm motility in order to select good quality dog semen samples after freezing and thawing.

Under our experimental conditions, greater survival in the female tract would be expected from frozen-thawed SLC-selected sperm, which showed a long sperm DNA longevity. This is in agreement with others authors (Imrat et al., 2012), who hypothesized that a slower sperm DNA fragmentation may be associated with greater sperm viability in the female genital tract. All these issues may indicate that the longevity of the DNA sperm should be a potential indicator of fertilizing ability of dog semen. Its analysis might be also interesting in all situations in which semen is handled for artificial insemination. Unfortunately, no fertility trials could be included in this study. Further studies, including fertility trials, are needed to investigate this hypothesis.

In conclusion, no effect of cryopreservation was observed on baseline values of dog sperm DNA fragmentation assessed with the SCDt. Additionally, SLC-selection using Androcoll-C improved longevity of frozen-thawed sperm DNA assessed after 24 h at physiological temperature of incubation using the SCDt.

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CHAPTER 3

Identification of sperm morphometric subpopulations in cooled-stored canine sperm and its relation with sperm DNA integrity

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Identification of sperm morphometric subpopulations in cooled-stored canine sperm and its relation with sperm DNA integrity

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Contents

The aims of this study were to (i) identify different morphometric subpopulations in cooled-stored canine sperm and their patterns of distribution during cool-storage for up to 240 hr and (ii) determine whether or not morphometric sperm subpopulations (sP) are related to sperm DNA integrity. For that purpose, morphometric parameters were analysed by computer-assisted sperm analysis (CASA) and sperm DNA fragmentation (sDFi) using the sperm Halomax test. Four morphometric sperm heads subpopulations were identified: sP1 (large and rounded), sP2 (large and elongated), sP3 (small and rounded) and sP4 (small and elongated). sP1 was the most predominant subpopulation for up to 72 hr and thereafter sP3 increased progressively. sDFi increased after 48 hr of cool-storage. Although sP3 showed a positive correlation with sDFi, and both increased over time, it could not be ensured that only the sperm with fragmented DNA are accumulated in sP3. In conclusion, sP3 and DNA fragmentation increased progressively during cool-storage, becoming possible indicators of sperm damage. However, it cannot be concluded that sP3 only contains sperm with fragmented DNA.

1 | INTRODUCTION

Computer-assisted sperm morphometry analysis systems provide a number of objective parameters and can be considered a powerful tool to improve the spermiogram (Núñez-Martinez, Moran, & Peña, 2005) based on their relationship with sperm quality and fertility (Casey, Gravance, Davis, Chabot, & Liu, 1997; Hirai et al., 2001; Ombelet, Menkveld, Kruger, & Steeno, 1995; de Paz et al., 2011). These parameters allow the identification of different subpopulations of spermatozoa within the mammalian ejaculate (Peña et al., 2005). Sperm subpopulations analysis has become an issue of considerable interest for the evaluation of sperm samples (Núñez-Martinez et al., 2005; Saravia et al., 2007). It has been hypothesized that their origin can be due to differences in the assembly of individual spermatozoa during spermatogenesis as well as to differential maturational status and age through mixing in the epididymis (Abaigar, Holt, Harrison, & Del Barrio, 1999). In this regard, the existence of sperm subpopulations based on both computer-assisted semen analysis (CASA)-derived kinematics (Dorado, Galvez, Murabito, Munoz-Serrano, & Hidalgo, 2011; Dorado, Alcaraz et al., 2011; Martinez-Pastor et al., 2005) and morphometric parameters (Álvarez et al., 2008; Lange-Consiglio et al., 2010; Núñez-Martinez et al., 2005) of the spermatozoa is now widely accepted by the scientific community (Peña, Núñez-Martínez, & Morán, 2006).

A number of studies have attempted to address the role of sperm head morphometry on the prediction of freezability (Álvarez et al., 2008; Núñez-Martinez, Moran, & Peña, 2007). In addition, it has been hypothesized that sperm head morphometric parameters may reflect chromatin organization, as the sperm head consists almost entirely of DNA (Lange-Consiglio et al., 2010). Due to that, subtle changes of sperm head can be related to abnormal DNA structure (Núñez-Martinez et al., 2007). Most of the studies published have analysed the sperm DNA using the sperm chromatin structure assay (SCSA). Since the SCSA was first described by Evenson, Darzynkiewicz, and Melamed (1980), several studies have been published in different species

(Álvarez et al., 2008; Evenson, 2013; Prinosilova, Rybar, Zajicova, & Hlavicova, 2012). Moreover, Fernández et al. (2003) proposed an alternative method for the determination of sperm DNA fragmentation (sDFi), the sperm chromatin dispersion test (SCDt). This simple, easy and cost-effective kit provides reliable results with a methodology that does not require a flow cytometer. The SCDt is commercialized for animal species with the commercial kit Sperm Halomax (Halotech DNA ® SL, Madrid, Spain). The basis of the technology lies on the differential response offered by the nuclei of spermatozoa with fragmented DNA compared to those with their intact DNA. The efficiency of SCDt to analyse the changes occurred in the sperm DNA has been previously demonstrated in different animal species (Cortes- Gutierrez et al., 2009; Fernandez et al., 2005; Urbano et al., 2013). To date, few studies have been able to find a relationship between sperm parameters and DNA integrity in canine sperm using the SCDt (Urbano et al., 2013).

Nowadays, the use of artificial insemination with shipped chilled dog semen has become very popular among breeders (Smith-Carr 2006). Commercial extenders commonly used for cool storage of dog spermatozoa at 5°C promise the maintenance of good sperm quality for at least 5 or even 10 days of storage (Ponglowhapan et al. 2004, Prinosilova et al., 2012). Some sperm parameters could be affected during cool storage which may affect sperm quality and fertility. In this sense, it has been shown by Hidalgo et al. (2015) that sDFi starts to increase after 2 days of cool storage in canine semen samples. A number of studies have been performed with cooled dog semen assessing different sperm parameters (Goericke-Pesch, Klaus, Failing, & Wehrend, 2012; Lopes, Simões, Ferreira, Martins-Bessa, & Rocha, 2009; Santana et al., 2013). However, to our knowledge, dog sperm morphometry during long-term cold storage and its relation with DNA fragmentation using the SCD test has not been evaluated yet.

This study was designed to (i) identify different morphometric subpopulations in cooled-stored canine sperm and their patterns of distribution during cool-storage and (ii) determine whether or not morphometric sperm subpopulations are related to sperm DNA integrity assessed by the SCDt.

2 | MATERIALS AND METHODS

2.1 | Animals

Four male dogs of different breeds (two Spanish Greyhounds, one German Shorthaired Pointer and one crossbreed) of unknown fertility were used. The ages of the dogs were from 4 to 5 years, and all of them were maintained with natural light, fed with commercial dry food and received water ad libitum. All dogs were trained for semen collection before the experiments. All animal procedures were performed in accordance with the Spanish laws for animal welfare and experimentation (RD 53/2013).

2.2 | Semen collection and evaluation

The sperm rich fraction of each ejaculate was collected into a calibrated pre-warmed plastic vial by digital manipulation as described by Linde-Forsberg (1995). A total number of twelve ejaculates (three per dog) were collected and pooled in four sampling occasions. Three different dogs were collected on each occasion, and the whole sperm rich fraction from each ejaculate was pooled to increase the volume and eliminate variability between donors (Verstegen, Onclin, & Iguer-Ouada, 2005). The four dogs were equally distributed through the pooled semen samples collected to ensure that each male contributed in the same number of sampling occasions (Hidalgo, Portero, Demyda-Peyrás, Ortiz, & Dorado, 2014). Before semen pooling, the presence of motile spermatozoa was always confirmed by visual examination of the sperm rich fraction of each ejaculate. Only semen samples with >70% of total motility were included in the study. The volume of each pooled semen sample was recorded and the sperm concentration measured using a sperm photometer (Spermacue Minitube, Tiefenbach, Germany).

2.3 | Semen processing

The semen samples were diluted 1:4 (v/v) in a commercial TRIS-based extender (Byladil A, Minitube, Tiefenbach, Germany) plus 20% egg yolk and conserved into a closed sterile vials. Samples were placed in the dark at room temperature (22°C) for 10 min and then gradually cooled (−0.3°C/min) to 5°C into a sperm container (Equitainer®, Minitüb, Tiefenbach, Germany) for 1 hr. Finally, the vials were put into a refrigerator and stored at 5°C for 10 days. An aliquot of each semen sample was taken for sperm analyses after 0, 24, 48, 72, 96, 168 and 240 hr of cool storage.

2.4 | Computer-assisted sperm morphometry analysis

Computer-assisted sperm morphometry analysis (CASMA) was assessed using the morphological module of the Sperm Class Analyzer (SCA v 5.4, Microptic SL, Barcelona, Spain). The features of the equipment have been described previously (Hidalgo, Rodriguez, Dorado, Sanz, & Soler, 2005). For that purpose, a 200 µl aliquot of each semen sample was mixed with 200 µl of phosphate-buffered saline (PBS D5773, Sigma-Aldrich, Spain). After that, sperm suspension was centrifuged for 5 min at 700 × *g* and the resulting sperm pellet was resuspended to a final concentration of 50 × 10^6 sperm/ml with PBS. Finally, 10 µl of sperm suspension was placed on the edge of a slide at 38°C and extended. Preparations were allowed to air dry for 30 min, stained with Diff-Quik® (Medium Diagnostics AG, Düdingen, Switzerland) as described previously (Hidalgo, Rodríguez, & Dorado, 2006) and then permanently sealed with Eukitt® mounting medium using a coverslip. At least 100 spermatozoa per slide were captured randomly in different fields with a bright-field 60x objective. This process was performed manually by interactive selection of sperm heads. When it was not possible to obtain a correct boundary of spermatozoa, the cells were eliminated.

Sperm heads were measured for four primary morphometric parameters: length (*L*), width (*W*), perimeter (*P*) in µm and area (*A*) in μm^2; three non-dimensional derived parameters of head shape: ellipticity

(L/W), rugosity ($4\pi A/P^2$) and elongation [$(L-W)/(L+W)$]; percentage of the sperm head occupied by the acrosome, the pale area delimited by the darker post-acrosomal region (Acro, %) and the intracellular grey level distribution (Grey, %).

2.5 | Sperm DNA fragmentation analysis

Sperm DNA fragmentation was assessed by the SCDt with the Sperm-Halomax commercial assay for canine semen (Halotech DNA SL, Madrid, Spain), following the manufacturer's instructions.

In brief, semen samples were diluted in PBS to a final concentration of $5x10^6$ sperm/ml. An aliquot of 25 μl of the sperm sample was added to a vial containing liquid low-melting agarose at 38°C. Then, a drop of the solution was placed on a pre-treated slide provided by the kit, covered with a coverslip (22 × 22 mm) and placed on a metallic plate, that had been previously cooled, for 5 min at 5°C. After that, the coverslip was carefully removed and the slide was immediately put into 10 ml of the lysis solution (in horizontal position) for 5 min at room temperature (22°C). Finally, the slide was washed for 5 min in distilled water, dehydrated in sequential 70, 90 and 100% ethanol baths (2 min each one) and air dried.

The slides were stained with 5 μl of propidium iodide (P4170-10MG, Sigma-Aldrich Quimica, S.L. Madrid, Spain), mounted with 5 μl of antifading solution (Vectashield, Vector Laboratories, Inc., Burlingame, USA), covered with a coverslip and sealed with nail polish. The slides were observed using a microscope (Olympus BX-40, Olympus U-RFL-T, Tokyo, Japan) provided for fluorescence using a U-ND25-2 filter (460–490 nm excitation filter). Five hundred sperm cells were counted in each slide. Spermatozoa with fragmented DNA showed a large halo of chromatin dispersion, and spermatozoa with intact DNA showed a small and compact halo of chromatin dispersion. The percentage of sperm with fragmented DNA (sDFi) was calculated in each semen sample and recorded for further analyses.

2.6 | Experimental design and statistical analysis

Statistic analyses were performed using the SPSS package, version 15.0 (SPSS Inc., Chicago, IL, USA) and the SAS statistic package v9.0 (SAS Institute Inc., Cary, NC, USA). Results were expressed as mean ± standard deviation (*SD*). The level of significance was set at $P < .05$.

2.6.1 | Experiment 1: effect of long-term cool storage on canine sperm morphometric subpopulations

A total of 2,837 spermatozoa were statistically evaluated to extract sperm subpopulations using the morphometric data obtained by means of clustering procedures as described previously by Núñez Martínez, Morán, and Peña (2006). The first step was to derive a small number of linear combinations (principal components [PCs]) that retain as much of the information in the original variables as possible. This allows to summarize many variables in few, jointly uncorrelated PCs. The VARIMAX method with Kaiser normalization was used as a rotation method. Then, the two-step cluster procedure was performed to indentify the different subpopulations present in the canine sperm using the sperm-derived parameters obtained after the principal component analysis.

The effect of cool-storage was evaluated on sperm morphometric subpopulations after 0, 24, 48, 72, 96, 168 and 240 hr of cool-storage. To analyse and compare the relative frequencies of spermatozoa belonging to each subpopulation and during cool-storage, chi-square test was used.

2.6.2 | Experiment 2: relationship between sperm head morphometry and sperm DNA fragmentation

This experiment was performed to assess the relationship between morphometric parameters and sperm DNA fragmentation index at each time of cool-storage. The relationship between each subpopulation and sDFi was also assessed. For that purpose, a regression analysis was conducted separately for each subpopulation, including a comparison of individual regression equations. Furthermore, the slopes of the regression equations of each subpopulation were compared using GraphPad Prism 6 (GraphPad Software, Inc., 7825 Fay Avenue, Suite 230, La Jolla, CA 92037 USA). Pearson correlation analysis was used to assess the relationship between DNA fragmentation index and mean values of sperm morphometric parameters or sperm subpopulations.

3 | RESULTS

3.1 | Experiment 1: effect of long-term cool storage on canine sperm morphometric subpopulations

Three PCs were identified with eigenvalues over 1, representing more than 94% of the cumulative variance (Table 1). Variables were grouped into those three PCs depending on the sperm head size (PC1: *L*, *W*, *A* and *P*), sperm shape (PC2: elongation, ellipticity, rugosity) and acrosome area and grey level (PC3) as shown in Table 2.

The two-step cluster procedure identified four sperm subpopulations: sP1, sP2, sP3 and sP4. Most of the sperm analysed were included into subpopulations 1, 3 and 4, while subpopulation 2 only included 107 spermatozoa. Table 3 shows the description of each subpopulation based on the mean values of morphometric parameters obtained. In this sense, sP1and sP2 showed higher values of *L*, *W* and low percentage of sperm head occupied by the acrosome, but sP1 contained spermatozoa with large rounded heads (low values of ellipticity) and sP2 enclosed spermatozoa with large elongated head (high values of ellipticity). On the other hand, sP3 and sP4 showed lower values of *L*, *W* and high or medium percentage of sperm head occupied by the acrosome, respectively; sP3 contained spermatozoa with small rounded heads (low ellipticity) and sP4 held spermatozoa with small elongated heads (high ellipticity).

The distribution of the four sperm subpopulations during cool storage was different (Figure 1). sP1 was the most abundant up to T72 followed by sP4 and sP3. At T96, sP1 and sP4 decreased

TABLE 1 Results of the principal components analysis (PCA) performed on the morphometric parameters assessed through the whole population of sperm analysed (n = 2,837).

	Initial eigenvalues		
Components	**Eigenvalues**	**% of the variance**	**Cumulative %**
1	3.76	41.74	41.74
2	3.00	33.37	75.10
3	1.76	19.56	94.66

TABLE 2 Matrix of components using the method of normalization Varimax

Sperm head morphometric parameters	Principal components		
	1	**2**	**3**
Length	**.86**	.49	.02
Width	**.87**	−.48	.05
Area	**.99**	−.04	.05
Perimeter	**.97**	.21	.05
Acrosome area	.08	−.09	**.95**
Grey level	.01	.05	**.95**
Elongation	−.06	**.98**	−.03
Ellipticity	−.06	**.99**	−.03
Rugosity	−.35	**−.84**	−.01

The bold numbers represented the main morphometric parameters belonging to each principal component.

and sP3 increased being the more predominant until T240. The proportion of sP2 was the lowest during the whole process of cool-storage.

Sperm DNA fragmentation index (sDFi) significantly increased (P < .01) after 48 hr of cool-storage (Figure 2).

3.2 | Experiment 2: relationship between sperm head morphometry and sperm DNA fragmentation

Correlation analysis showed no relationship between sperm DNA fragmentation and sperm morphometric parameters, with the exception of the percentage of the acrosome (Table 4). However, when we considered the different sperm subpopulations instead of mean values of morphometric parameters, we found a relationship between DNA fragmentation and sperm subpopulations (Figure 3). There was a moderate positive correlation (r = .46; P < .01) between DNA fragmentation index and subpopulation 3 (Figure 3c). Additionally, a low negative correlation (r = −.38; P < .05) was observed between DNA fragmentation index and subpopulation 1 (Figure 3a).

The trendlines of the four subpopulations during cool-storage (240 hr) are represented in Figure 4a. Subpopulation 3 showed a trendline with a positive slope while subpopulations 1, 2 and 4 had negative slopes. Significant differences were observed in the slopes between subpopulation 3 and the other subpopulations. Both sP3 and sDFi trendlines had positive but significantly different slopes (Figure 4b).

TABLE 3 Morphometric characteristics of each sperm morphometric subpopulation (n = 2,837)

		Morphometric parameters							
Subp	***n* (%)**	***L* (μm)**	***W* (μm)**	***A* (μm²)**	***P* (μm)**	**Acro (%)**	**Grey (%)**	**Elong**	**Elip**	**Rugo**
sP1	1090 (38.42)	5.84 ± 0.28^{b}	3.94 ± 0.18^{a}	19.31 ± 1.29^{b}	16.81 ± 0.63^{b}	66.76 ± 27.38^{b}	128.9 ± 50.21^{b}	0.19 ± 0.02^{c}	1.48 ± 0.07^{c}	0.86 ± 0.02^{b}
sP2	107 (3.77)	6.60 ± 0.41^{a}	3.72 ± 0.30^{b}	20.08 ± 2.01^{a}	18 ± 1.45^{a}	66.23 ± 16.93^{b}	139.99 ± 28.92^{a}	0.28 ± 0.04^{a}	1.78 ± 0.15^{a}	0.78 ± 0.06^{d}
sP3	862 (30.38)	5.27 ± 0.28^{d}	3.56 ± 0.21^{c}	16.15 ± 1.53^{d}	15.23 ± 0.78^{d}	74.94 ± 18.68^{a}	143.33 ± 25.89^{a}	0.19 ± 0.02^{c}	1.48 ± 0.07^{c}	0.87 ± 0.02^{a}
sP4	778 (27.42)	5.75 ± 0.28^{c}	3.48 ± 0.20^{d}	17.12 ± 1.49^{c}	16.02 ± 0.74^{c}	68.42 ± 15.28^{b}	140.99 ± 23.51^{a}	0.25 ± 0.02^{b}	1.65 ± 0.07^{b}	0.84 ± 0.02^{c}

Subp, subpopulations; sP, subpopulation; n, number of spermatozoa analysed (percentage of the whole sperm population belonging to each subpopulation is given in brackets); L, head length; W, head width; A, head area; P, head perimeter; Acro, percentage of the sperm head occupied by the acrosome; Grey, grey level; Elong, elongation; Elip, ellipticity; Rugo, rugosity.
Different letters indicate significant differences between sperm subpopulations for each morphometric parameter (P < .001).

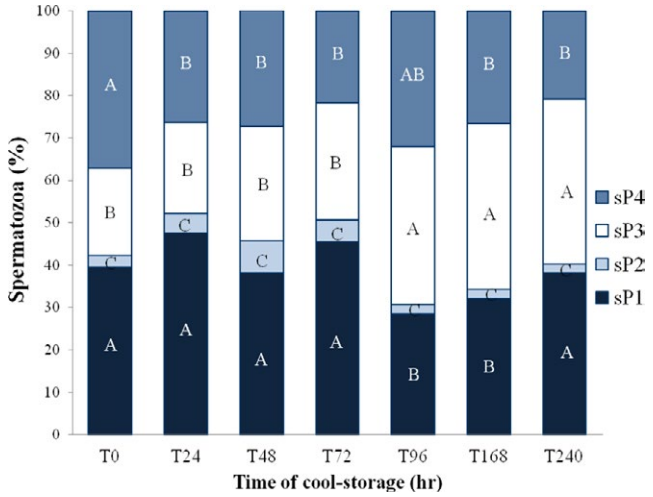

FIGURE 1 Distribution of the four sperm morphometric subpopulations identified at each time of cool-storage

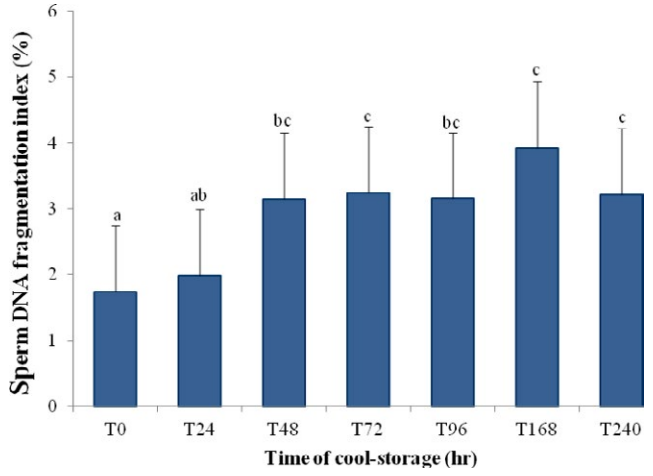

FIGURE 2 Sperm DNA fragmentation index (sDFi) during cool-storage (240 hr). Different letters indicate significant differences on the sperm DNA fragmentation index during cool-storage ($P < .01$)

4 | DISCUSSION

The present study has revealed the presence of four morphometric sperm subpopulations with different response to cooling process, based on the changes in their patterns of distribution during cool-storage, and its relationship with sperm DNA integrity.

Previous studies have demonstrated the existence of sperm morphometric subpopulations in fresh and frozen-thawed dog sperm (Núñez-Martinez et al., 2007; Rijsselaere, Van Soom, Hoflack, Maes, & de Kruif, 2004); however, to the best of the author's knowledge, changes in the distribution of morphometric dog sperm subpopulations during long-term cool-storage has not been determined yet. In this study, a large number of sperm were analysed from pooled semen samples to avoid male-to-male variability (Dorado et al., 2016; Verstegen et al., 2005), as differences between individuals can lead to erroneous results when different treatments are compared.

In the first experiment, the cluster analysis showed four sperm subpopulations with different morphometric features in cooled-stored semen samples. Núñez-Martinez et al. (2007) described five sperm subpopulations. However, in that study fresh semen was evaluated and one subpopulation included a low number of sperm which seems not able to survive to the cooling process. Differences in the number of subpopulations between studies could be due to the effect of cooling on the sperm heads. In the present study, the distribution of the proportions of the four subpopulations showed fluctuations during long-term cool-storage (240 hr). Similar results have been described by González Villalobos et al. (2008) and more recently by Morales, Quintero-Moreno, Osorio-Meléndez, and Rubio-Guillén (2012) in boar sperm showing that some morphometric characteristics of the sperm head can be affected by the cooling process. In addition, González Villalobos et al. (2008) suggested that these changes might be caused by the slight deterioration of the ejaculate sample, due to the oxidative stress.

In this sense, sperm cooling affects the physical properties of all cell membranes (Peña, Johannisson, Wallgren, & Rodriguez Martinez, 2004; Quinn, 1989). It is known that the differences on the biochemical composition of the sperm plasma membrane and, more specifically, the amount of phospholipids and cholesterol, are related to the sensibility of the spermatozoa to the cooling process. Considering our results, we can assume that sP1 is the most stable subpopulation during the first 72 hr of cool storage. sP1 contains spermatozoa with large rounded heads, and those spermatozoa in canine ejaculates should respond better to the structural changes during cool-storage. In contrast, sP3 contained small rounded spermatozoa which increased progressively after 72 hr of cool storage while

TABLE 4 Correlation analysis between sperm DNA fragmentation and mean values of sperm morphometric parameters assessed across the whole population of sperm analysed (n = 2,837)

	Morphometric parameters								
	L (μm)	*W* (μm)	*A* (μm2)	*P* (μm)	Acro (%)	Grey (%)	Elong	Elip	Rugo
sDFi (%)	−0.35	−0.22	−0.31	−0.32	0.50	0.07	−0.18	−0.16	0.29
P-value	0.06	0.25	0.11	0.09	0.01	0.73	0.37	0.41	0.14

sDFi, sperm DNA fragmentation index; *L*; head length, *W*; head width, *A*; head area, *P*; head perimeter, Acro; percentage of the sperm head occupied by the acrosome, Grey; grey level, Elong; elongation, Elip; ellipticity, Rugo; rugosity.

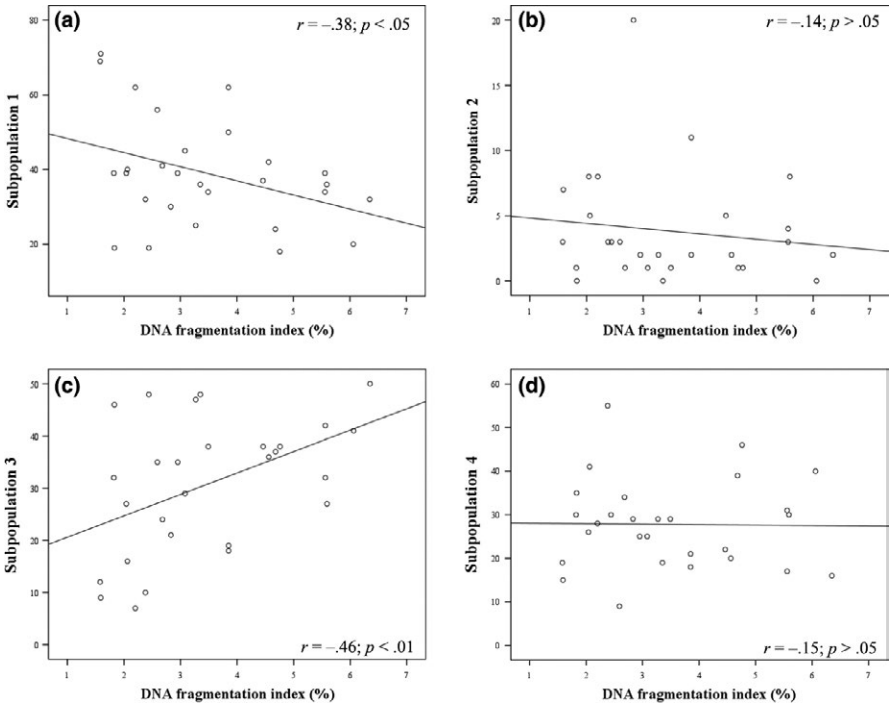

FIGURE 3 Relationship between sperm DNA fragmentation index and each of the four morphometric sperm subpopulations identified

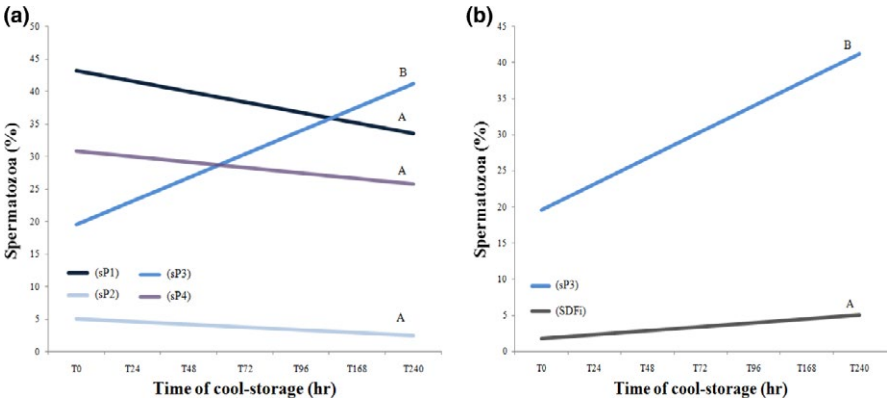

FIGURE 4 Regression analysis of morphometric sperm subpopulations and DNA fragmented sperm during 240 hr of cool-storage: (a) comparison of slopes between sperm subpopulations; (b) comparison of the slope of subpopulation 3 and sperm with DNA fragmented. Different letters between trendlines indicate significant differences ($P < .05$)

subpopulations 1, 2 and 4 decreased, maybe because those sperm are transformed into subpopulation 3 during cool-storage, which is the most predominant after 240 hr, indicating damage due to cooling process.

On the other hand, sperm head morphometry has been previously used to predict cryopreservation success (Álvarez et al., 2008; Esteso, Fernández-Santos et al., 2006; Núñez-Martinez et al., 2007). It is well known that cryopreservation involves major changes in sperm cell environment (Mazur & Cole, 1989). In this sense, changes in sperm head morphometry due to cryopreservation have been widely related with small dimensions of sperm heads after thawing. Esteso, Soler, Fernández-Santos, Quintero-Moreno, and Garde (2006) evaluated head morphometric size and shape for determining cryoresistance in red deer sperm, describing spermatozoa with small, elongated and narrow head as "good" freezer and spermatozoa with big short and width head as "bad" freezer. In the present study, sP1 (large and rounded sperm) was predominant for up to 72 hr of cooling, from that time on, sP1 percentage decreased and sP3 (small and rounded) percentage increased. It seems that cooling for longer periods than 72 hr shrinks sperm heads as was described for cryopreservation in other species. This shrinking may reflect the negative effect of cool-storage on canine spermatozoa.

In the second experiment, the relationship between sperm head morphometry and sperm DNA integrity was analysed. Some studies have demonstrated the relationship between changes of sperm head shape and DNA integrity (Álvarez et al., 2008; Núñez-Martinez et al., 2007; Utsuno, Oka, Yamamoto, & Shiozawa, 2013). These studies used techniques such as SCSA or terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) to assess the sperm DNA fragmentation. In this study, we used the SCDt to assess sperm DNA fragmentation index. Firstly, the relationship between DNA fragmentation index and the mean values of sperm morphometric parameters was assessed. No relationship was found, except to the percentage of head occupied by acrosome. Similar results were found by Vernocchi,

Morselli, Lange Consiglio, Faustini, and Luvoni (2014) using SCDt to relate DNA fragmentation and sperm head morphometry in cat epididymal spermatozoa, showing that DNA fragmentation seemed to be independent from the other measured morphometric variables (using mean values of morphometric parameters). In contrast, Álvarez et al. (2008) demonstrated significant correlations between SCSA and CASMA parameters in brown bear (*Ursus arctos*) spermatozoa. Maybe, these differences could be related to the fact of using different methods to assess sperm DNA integrity, namely SCSA and SCDt. Although both techniques are useful for studying sperm DNA fragmentation, they explain different aspects of sperm DNA damage, as the lysis solution used in the SCD test alters the chromatin in a different manner than the acid detergent used in SCSA, causing a disintegration of the protein scaffold, as explained by Martínez-Pastor, Del Rocío Fernández-Santos, Domínguez-Rebolledo, Esteso, and Garde (2009).

Secondly, relationship between sperm DNA fragmentation index and the four sperm morphometric subpopulations was evaluated. In this way, a similar distribution between DNA fragmentation index and morphometric subpopulation 3 (spermatozoa with small rounded heads) was found. Similar results were described by Núñez-Martinez et al. (2007), finding that high DNA denaturation was related to short and wide sperm heads in fresh canine sperm. If we consider that cryopreservation reduces the sperm head dimensions as described above (Esteso, Fernández-Santos et al., 2006), our results could be indicating the same changes during cool-storage, with a subpopulation of small and rounded sperm (sP3). Hence, this could help to understand the behaviour of the different sperm morphometric subpopulations with respect to the DNA damage.

In this study, DNA fragmentation index increased after 48 hr of cool storage. Similar results were found by Hidalgo et al. (2015) evaluating the DNA integrity in cooled dog sperm using the SCDt. In addition, both sP3 and sperm DNA fragmentation increased progressively during cool-storage, showing a positive correlation. However, it is difficult to relate sP3 with sperm with fragmented DNA. Firstly, their rates of increment (slopes) are significantly different. Secondly, sP3 ranged from 20% to 40% and sDFi form 2.5%–5%. Thus, it is not possible to ensure that only the sperm with fragmented DNA are accumulated in sP3. Nevertheless, as sP3 (small and rounded sperm heads) and DNA fragmentation index (sDFi) increase during cool storage, both could be indicators of sperm damage.

In conclusion, four sperm morphometric subpopulations were identified, but only sP3 (small and rounded sperm heads) increased progressively after 72 hr of cool-storage, becoming a possible indicator of sperm damage. Although there was a positive correlation between sP3 and DNA fragmentation, it cannot be concluded that sP3 only contains sperm with fragmented DNA.

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CONFLICT OF INTEREST

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

AUTHORSHIP CONTRIBUTION

M Hidalgo and M Urbano contributed to all sections. I Ortiz and J Dorado contributed to the study design, data analysis and interpretation, preparation of the manuscript and final approval of the manuscript.

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CHAPTER 4

Comparison of DNA fragmentation of frozen-thawed epididymal canine sperm using SCSA and SCDt

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Comparison of DNA fragmentation of frozen-thawed epididymal canine sperm using SCSA and SCDt

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Running title: DNA fragmentation on frozen-thawed epididymal canine sperm

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Abstract

The aim of this study was to compare the sperm DNA fragmentation of frozen-thawed epididymal canine sperm using the SCSA (Sperm Chromatine Structure Assay) and SCDt (Sperm Chromatin Dispersion test). For this purpose, epididymis from neutered dogs were minced and incubated in a Tris-based extender. The recovered sperm were slowly cooled and frozen and stored in liquid nitrogen. After thawing, each replica was incubated at 38 ºC for 24 h. Sperm DNA fragmentation index (sDFi) was assessed by SCSA and sCDt at 0, 3, 6 and 24 h of incubation and compared within treatments by ANOVA. The relationship and agreement between techniques were evaluated by Pearson's coefficient and Intraclass Correlation Coefficient (ICC). The results were expressed as mean ± standard error of the mean (SEM). Both techniques showed a significant increase of DNA fragmentation after 24 h of incubation. Moderate correlation ($r=0.65$; $P<0.01$) but poor agreement (ICC=0.451; $P>0.05$) was found between SCSA and SCDt. The lack of agreement indicates that SCSA and SCDt measure different aspects of DNA fragmentation. Four halo morphologies were found after 24 h of incubation using the SCDt: unfragmented DNA with a small halo, fragmented DNA with large halo and two new halo presentations never described before for dog sperm: receding sperm with a disappearing halo and bald sperm without chromatin dispersion halo around the core. Bald sperm are not described by the manufacturer and they are similar to unfragmented sperm, which could lead to erroneous results in the SCDt. Further studies with different incubation periods and considering the new morphologies described in this study should be performed. In conclusion, although SCSA and SCDt can evaluate the changes in the sperm DNA

fragmentation dynamics of frozen-thawed epididymal dog sperm, they yielded different information on sperm DNA fragmentation.

Keywords: cryopreservation; dog; epididymis; sperm DNA fragmentation; SCSA; SCD.

1. Introduction

Cryopreservation of sperm collected from the epididymis allows the use of genetic material post-mortem or after orchiectomy from high value animals or endangered species (Thomassen and Farstad, 2009). During the transit along the epididymis, spermatozoa suffer morphological and functional changes as part of its maturation process, which allows them to acquire progressive motility and fertilising capacity (Fouchecourt et al., 2000). Chromatin condensation begins during spermiogenesis, when the histones are first replaced by transition proteins and finally by small, basic protamines. These protamines form inter- and intramolecular disulfide bonds to fully condense chromatin. The site in the epididymis where the main condensation takes places in the dog is the caput-corpus transit, from this point on, the degree of DNA condensation is very similar to the ejaculate (Grimes Jr et al., 1997).

In this regard, sperm DNA fragmentation can be evaluated using different techniques (Chohan et al., 2006a; Ribas-Maynou et al., 2013; Evenson, 2016). The sperm chromatin structure assay (SCSA) has been widely used to assess sperm DNA fragmentation in human and other mammalian species (Chohan et al., 2006b). The sperm chromatin dispersion test (SCDt) is a relatively new

technique reported to assess DNA fragmentation firstly in human sperm (Fernández et al., 2003) and then adapted mammalian species (Enciso et al., 2006; Lopez-Fernandez et al., 2008; Cortes-Gutierrez et al., 2009; Gosálvez et al., 2011). In dogs, sperm DNA integrity has been evaluated in fresh (Núñez-Martinez et al., 2005; Lange-Consiglio et al., 2010; Urbano et al., 2013), cooled (Hidalgo et al., 2015) and frozen-thawed (Urbano et al., 2013) semen samples; however, there are few reports that assessed DNA integrity on epididymal canine sperm (Garcia-Macias et al., 2006; Varesi et al., 2014), and none of them evaluates DNA fragmentation dynamics. Moreover, SCSA and SCDt have been successfully used to evaluate canine sperm DNA fragmentation separately (Núñez-Martinez et al., 2005; Hidalgo et al., 2010; Prinosilova et al., 2012; Urbano et al., 2013; Varesi et al., 2014; Hidalgo et al., 2015); but their results have not been compared yet.

Therefore, the aim of this study was to compare the sperm DNA fragmentation of frozen-thawed epididimal canine sperm using the SCSA and SCDt.

2. Materials and methods

2.1. Collection of epididymal sperm

Sperm were obtained from pairs of epididymidis from six clinically healthy dogs of different breeds (three Spanish greyhounds, one Andalusian Rat-Hunting, one crossbreed and one Andalusian hound), aged 2-8 years. Animals were provided by private owners. Testes with the attached epididymidis were obtained after routine castration at the Veterinary Teaching Hospital of the University of Cordoba, Spain. All animal procedures were performed in

accordance with the Spanish laws for animal welfare and experimentation (RD 53/2013).

Immediately upon removal, the testes (with ligated vas deferens) were placed into physiological saline at room temperature (≈22ºC) and stored at 5ºC for 1-2 hours before sperm processing. After that, the whole epididymis, including part of the proximal vas deferens, was dissected free from the testis. Epididymis were placed in 35-mm plastic culture dishes with 1ml of Tris extender and were minced with surgical scalpels and scissors. The minced epididymis were incubated in Tris-based extender (3.025g of Tris, 1.7g of citric acid, 1.25g fructose, 0.06g benzyl-penicillin, 0.1g Streptomycin per 100ml) for 30 minutes at 38 ºC. After that, the extender containing the spermatozoa was placed into a calibrated plastic tube.

2.2. Semen processing

The concentration of semen samples was determined using the Sperm Class Analyzer (SCA, Microptic SL, Spain). Then, the sperm samples were centrifuged (700g/5minutes) and the sperm pellet diluted in Tris-based extender, adding 20% egg yolk (v/v) and 3% glycerol, to obtain 100 x 10^6 sperm/mL. Sperm samples were placed in the dark at room temperature for 10 minutes and then gradually cooled to 5ºC (-0.3ºC/minute) into a sperm container (Equitainer®, Minitüb, Tiefenbach, Germany) for to 2 hours. Thereafter, an equal volume of a second Tris-based extender (at 5ºC), containing 20% egg yolk but 7% glycerol was added, to obtain a final concentration of 50 x 10^6 sperm/ml. Immediately, 0.5 ml straws were filled with the diluted sperm and sealed (Ultraseal 21™, Minitube, Tiefenbach, Germany).

The straws were horizontally frozen in nitrogen vapours for 10 minutes at 4 cm from the surface of liquid nitrogen and then stored in liquid nitrogen tanks. Straws were thawed in a water bath at 37 ºC for 30 seconds. Sperm DNA fragmentation was assessed at 0, 3, 6 and 24 hours of incubation at 38ºC as described below.

2.3. Assessment of sperm DNA fragmentation using the Sperm Chromatin Dispersion test (SCDt)

Sperm DNA fragmentation was assessed using the Sperm-Halomax® commercial assay (Halotech DNA SL, Madrid, Spain), designed for canine semen. Semen samples were diluted in phosphate buffer solution (PBS, Sigma-Aldrich Chemie GmbH, Steinheim, Germany) to a final concentration of 5 x 10^6 sperm/mL. An aliquot of 25 µL was added to a vial containing 50 µL liquid low-melting agarose at 38ºC. This vial was previously immersed in a water bath at 90-100 °C for 5 minutes and subsequently was left to warm for a further 5 minutes at 38 ºC. Then, a drop of the sperm agar mixture was placed on a pretreated slide provided by the kit and covered with a 22x22mm coverslip. The preparation was placed on a cooled metal plate and then stored in a refrigerator at 5° C for 5 minutes. After that, the coverslip was carefully removed and the slide was covered (in horizontal position) with lysis solution (containing β-mercaptoethanol) for 5 minutes at room temperature (≈22ºC). Subsequently, the preparation was washed and covered with distilled water for 5 minutes and then immersed in three successive baths of ethanol at 70%, 90% and 100% for 2 minutes each. Finally, the slides were allowed to air-dry before staining. The slides were stained with 1µl of SYBR® Green I (S9430, Sigma-Aldrich Sweden

AB, Stockholm, Sweden), mounted with 1µl of antifading and a coverslip. The slides were observed using an epi-fluorescence microscope (Dialux 20; Leitz Wetzler, Germany). Three hundred sperm were counted in each slide. Spermatozoa with fragmented DNA showed a large halo of spotty halo of chromatin dispersion and spermatozoa with unfragmented DNA only show a small and compact halo of chromatin dispersion.

2.4. Assessment of sperm DNA fragmentation using the Sperm Chromatin Structure Assay (SCSA)

Sperm DNA fragmentation was assessed using the FacStarPLUS flow cytometer (Becton Dickinson, San Jose, CA, USA). Sperm samples were mixed with TRIS–sodium–EDTA (TNE) buffer (0.15 M NaCl, 0.001 M TRIS, and 0.001 M 2Na-EDTA, pH 6.8) to 1-2 x 10^6 sperm/mL. Thereafter, 500µl of TNE buffer was mixed with 10µl of the sperm sample in a cryotube, snap-frozen on liquid nitrogen and stored to -80ºC until it was required for flow cytometry. Then, 200 µl of this suspension were treated with 200 µl of an acid-detergent solution (0.08 N HCl, 0.1% Triton X-100, pH 1.2) for exactly 30 seconds. After that, 600 µl of an AO staining solution (Sigma–Aldrich, St. Louis, MO, USA) (6 µg/mL in phosphate–citrate buffer) was added and the sample was placed in the flow cytometer for SCSA analysis. A total of 10,000 sperm were analyzed in two replicates for each epididymal sperm sample. Data were evaluated using FCS Express (Express Flow Cytometry Data Analysis), version 2 (De novo Software, Thornhill on Canada).

2.5. Statistical analysis

Normality of the data distributions was checked by the Kolmogorov- Smirnov test. The results were expressed as mean ± standard error of the mean (SEM). The effect of incubation time on DNA integrity of canine epididymal sperm was evaluated by ANOVA followed by the Tukey pos-hoc test. Comparisons between methods at each time of incubation (T0, T3, T6 and T24) were performed using the Student´s t-test. Correlation analysis (SCSA vs. SCDt) was performed using the Pearson coefficient (r value) and was represented in a scatter plot. The agreement of the results obtained with both methods was assessed using the intra class correlation coefficient (ICC). All statistical analyses were performed using SPSS v15.0 (SPSS Inc., Chicago, IL, USA). The level of significance was set at $P<0.05$.

3. Results

No significant differences were found in the sperm DNA fragmentation index (sDFi) assessed with each method during the first 6 hours of incubation. However, after 24 hours of incubation, both techniques showed a significant increase of DNA fragmentation (Table 1). After the dynamic analysis of sperm DNA fragmentation, four sperm morphologies were founded: the first one including a small halo of chromatin dispersion around the sperm core (Fig. 1a), the second one present a large halo of chromatin dispersion with a highly degraded sperm core (Fig. 1b), the third one show a “receding-halo” sperm with disappearing halo around the sperm core (Fig. 1c) and the last one showing a compact core but without halo of chromatin dispersion (Fig. 1c). The Pearson analysis showed a significant moderate positive correlation ($r=0.65$; $P<0.01$,

193 Fig. 2) between methods but the Intraclass Correlation Coefficient (ICC=0.451;
194 P>0.05) showed no good agreement in the measurements obtained.

195

196 **4. Discussion**

197 The aim of this study was to elucidate if SCSA and SDCt give the same
198 information on DNA fragmentation of frozen-thawed epididymal canine sperm.
199 The differences between sDFi of ejaculated and epididymal sperm was already
200 study by Garcia-Macias et al. (2006), who concluded that the critical point
201 regarding chromatin condensation assessed by SCSA was located in the
202 transition between the caput and the corpus epididymis. Even though in this
203 study the whole epididymis was minced to obtain as many sperm as possible
204 sDFi values remain similar to those from frozen-thawed ejaculated samples
205 (Urbano et al., 2013). On the other hand, a previous study (Varesi et al., 2014)
206 showed that there were not differences between fresh and frozen-thawed dog
207 epididymal sperm using the SCDt. However, both studies evaluated DNA
208 fragmentation by static analysis, this type of analysis does not allow to evidence
209 the cryptic damage in the DNA molecule and responsible for many false
210 negative results (Gosálvez et al., 2011). Thus, frozen-thawed samples were
211 submitted to a stressor (incubation at 37 °C for 24 h) to perform a dynamic
212 assessment. Significant differences were found in sDFi values assessed by
213 both methodologies (SCSA and SCDt) only after 24 h of incubation, which is in
214 agreement with other studies performed in ejaculated frozen-thawed semen
215 samples (Urbano et al., 2013).

At first glance, these results could indicate that both techniques evaluate sDFi similarly, nevertheless, when looking at the results carefully we can appreciate that SCSA shows higher sDFi values than SCDt, particularly after 24 h of incubation. This finding made us delve into the possible relationship between SCSA and SCDt. In this regard, a very significant ($P<0.01$) correlation was found between techniques, unfortunately, correlation only describes the degree (moderate, $r=0.65$) and direction (positive) of a relationship, but does not measure the actual difference between tests (Foster et al., 2011). In this sense, the Intraclass Correlation Coefficient (ICC) is a measure of the reliability of measurements. In our study, the ICC (0.451) was lower than 0.7, which means that the sDFi values obtained by SCSA and SCDt are not consistent. It seems now obvious, as it was already shown in other species (De La Torre et al., 2007; Martínez-Pastor et al., 2009), that SCSA and SCDt do not equally evaluate DNA fragmentation. In order to understand the differences between techniques it is needed to address the fundamental basis of both techniques. On the one hand, both methods are based on the resistance to iatrogenic denaturalization of the chromatin molecule, nevertheless, the mechanism of action is completely different. SCSA uses hydrochloric acid and Triton X (a detergent) to extract the protamines which in turn bring DNA as well. SCDt uses β-mercaptoethanol, a reducing agent that affects the disulfide bonds and destroy the native conformation of the protamines. It seems logical that the longer the incubation period, the bigger differences we will see between techniques. Moreover, unlike SCDt is species-specific, SCSA was designed for human beings and uses the same lysis solution concentration, times and equipment for all the species. Therefore, further studies are needed to assess

241 which denaturalization protocol resembles better the physiological damage in
242 the DNA molecule.

243 On the other hand, after the denaturalization of the molecule, SCSA and
244 SCDt evaluate the DNA fragmentation differently. SCSA evaluates sDFi by
245 laser. Although it might seem a more objective valuation of the damage, the
246 interspecific differences can lead to some alterations in the analysis, such as
247 the differences in the geometry of the dog sperm head (smaller and shorter)
248 would render different fluorescence depending on laser incidence (Garcia-
249 Macias et al., 2006). In this sense, a skilled operator in DNA analysis should
250 fine-tune the software to determine which population of the scattergram belongs
251 to low, medium or high fragmented sperm DNA, fact that makes the technique
252 not completely objective. In the SCDt, an operator should count sperm manually
253 and differentiate two different morphologies: the halo is not as large as twice the
254 diameter of the core (non-fragmented DNA; Fig. 1a), or the halo is at least twice
255 the diameter of the core (fragmented DNA; Fig. 1b). Surprisingly, after 24 h of
256 incubation, we found not two but four different sperm morphologies with SCDt;
257 the two classic halo presentations, already described above, and two new
258 morphologies, which have never been described before in dog semen:
259 spermatozoa with a “receding-halo” with a loose halo around the sperm core
260 (identified by us as “receding” sperm; Fig. 1c) and spermatozoa showing a
261 compact core but without halo of chromatin dispersion (described by us as
262 “bald” sperm; Fig. 1d). Similarly, Pérez-Llano et al. (2010) after performing a
263 dynamic analysis of sperm DNA damage in boar semen described “bald”
264 sperm, showing a compact core lacking halo around, as described in this study.
265 This phenomenon was explained using the comet assay, evidencing that bald

sperm have fragmented DNA in which there is such damage in the protamines that they drag the chromatin away from the core, seeming as if they have no halo and resembling unfragmented DNA according to the manufacturer's description, which could lead to erroneous results. This fact would explain the lower values obtained in SCDt in comparison to SCSA after 24 h of incubation. Therefore, in further studies these findings should be taking into account, with shorter incubation periods (longer than 6 and shorter than 24 h) and contemplating 4 sperm morphologies (unfragmented DNA with a small halo, and fragmented DNA with either large halo or receding or bald sperm). In short, since SCSA and SCDt denature the chromatin molecule and evaluate the damage differently, it is reasonable to think that they yield different information on sperm DNA fragmentation.

Acknowledgments

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CONCLUSIONES

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According to the results obtained in each publication, we conclude the following:

CONCLUSION 1 (First publication): *DNA integrity of canine spermatozoa during chill storage assessed by the sperm chromatin dispersion test using bright-field or fluorescence microscopy.* M Hidalgo, M Urbano, I Ortiz, S Demyda-Peyras, MR Murabito, MJ Gálvez, J Dorado (2015).

Sperm-Halomax, developed for canine semen and based on the SCDt, can be used to assess the sperm DNA integrity of chilled-stored canine semen using both bright-field and fluorescence microscopy, and the sDFI increased after 48 hours of chill storage, which could detect sperm damage earlier than other routine sperm parameters, such as sperm motility.

CONCLUSION 2 (Second publication): *Effect of cryopreservation and single layer centrifugation on canine sperm DNA fragmentation assessed by the sperm chromatin dispersion test.* M Urbano, J Dorado, I Ortiz, JM Morrell, S Demyda-Peyras, MJ Gálvez, L. Alcaraz, L Ramírez, M Hidalgo (2013).

No effect of cryopreservation was observed on baseline values of dog sperm DNA fragmentation assessed with the SCDt, and SLC-selection using Androcoll-C improved longevity of frozen-thawed sperm DNA assessed after 24 hours of incubation at physiological temperature using the SCDt.

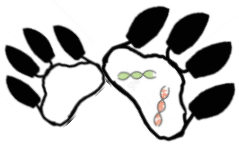
CONCLUSION 3 (Third publication): *Identification of sperm morphometric subpopulations in cooled-stored canine sperm and its relation with sperm DNA integrity.* M Urbano, I Ortiz, J Dorado, M Hidalgo (2017).

Four sperm morphometric subpopulations were identified, but only sP3 (small and rounded sperm heads) increased progressively after 72h of cool-storage, becoming a possible indicator of sperm damage; although there was a positive correlation between sP3 and DNA fragmentation, it cannot be concluded that sP3 only contains sperm with fragmented DNA.

CONCLUSION 4 (Fourth publication): *Comparison of DNA fragmentation of frozen-thawed epididymal canine sperm using SCSA and SCDt.* I Ortiz, M Urbano, J Dorado, JM Morrell, E Al-Essawe, A Johannisson, M Hidalgo.

Although SCSA and SCDt can evaluate the changes in the sperm DNA fragmentation dynamics of frozen-thawed epididymal dog sperm, they yielded different information on sperm DNA fragmentation.

CONCLUSIONS



CONCLUSIONES

CONCLUSIONES

De acuerdo a los resultados obtenidos en cada una de nuestras publicaciones, nosotros concluimos lo siguiente:

CONCLUSIÓN 1 (Primera publicación): *DNA integrity of canine spermatozoa during chill storage assessed by the sperm chromatin dispersion test using bright-field or fluorescence microscopy.* M Hidalgo, M Urbano, I Ortiz, S Demyda-Peyras, MR Murabito, MJ Gálvez, J Dorado (2015).

> El test Halomax, creado específicamente para el semen canino, y basado en el test de dispersión de la cromatina espermática, puede emplearse para valorar la integridad del ADN espermático del semen refrigerado de perro utilizando microscopía de campo claro y de fluorescencia, y el índice de fragmentación del ADN espermático aumenta a las 48 horas de refrigeración, por lo que podría detectar el daño espermático más tempranamente que otros parámetros espermáticos de rutina, como el movimiento espermático.

CONCLUSIÓN 2 (Segunda publicación): *Effect of cryopreservation and single layer centrifugation on canine sperm DNA fragmentation assessed by the sperm chromatin dispersion test.* M Urbano, J Dorado, I Ortiz, JM Morrell, S Demyda-Peyras, MJ Gálvez, L. Alcaraz, L Ramírez, M Hidalgo (2013).

No se observó ningún efecto en la fragmentación del ADN espermático del perro tras la criopreservación con el test de Dispersión de la Cromatina Espermática (SCDt), y la selección coloidal en una sola capa usando Androcoll-C mejora el esperma congelado de perro cuando es incubado a temperatura fisiológica durante 24 horas y es valorado con el test de Dispersión de la Cromatina Espermática (SCDt).

CONCLUSIÓN 3 (Tercera publicación): *Identification of sperm morphometric subpopulations in cooled-stored canine sperm and its relation with sperm DNA integrity*. M Urbano, I Ortiz, J Dorado, M Hidalgo (2017).

Cuatro subpoblaciones morfométricas fueron identificadas en el esperma refrigerado de perro, pero solo la subpoblación 3 (espermatozoides con cabezas pequeñas y redondas) aumentó progresivamente tras 72 horas de refrigeración, llegando a ser un posible indicador del daño espermático; aunque hemos encontrado una correlación positiva entre la subpoblación 3 y la fragmentación del ADN espermático, no podemos concluir que la subpoblación 3 sólo contenga espermatozoides con el ADN fragmentado.

CONCLUSIÓN 4 (Cuarta publication): *Comparison of DNA fragmentation of frozen-thawed epididymal canine sperm using SCSA and SCDt.* I Ortiz, M Urbano, J Dorado, JM Morrell, E Al-Essawe, A Johannisson, M Hidalgo.

Aunque el SCSA y el SCDt pueden evaluar los cambios dinámicos en la fragmentación del ADN del espermatozoide congelado de epidídimo de perro, ambos ofrecen una información diferente sobre la fragmentación del ADN.

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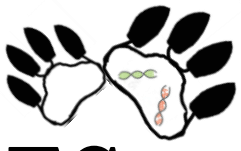

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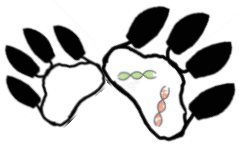
QUALITY INDEXES



ÍNDICES DE CALIDAD

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PRIMERA PUBLICACIÓN:

- Título: DNA integrity of canine spermatozoa during chill-storage assessed by the sperm chromatin dispersion test using bright field or fluorescence microscopy

- Autores (p.o. de firma): M Hidalgo, M Urbano, I Ortiz, S Demyda-Peyras, MR Murabito, MJ Gálvez, J Dorado

- Revista (año,vol.,pág.): Theriogenology 84 (2015) 399-406

- Base de Datos Internacional o Nacional en las que está indexada: Journal of Citation Reports (JCR). 2015.

- Área temática en la Base de Datos de referencia: Veterinary Sciences

- Índice de impacto de la revista en el año de publicación del Artículo: 1,838

- Lugar que ocupa/Nº de revistas del Área temática: 18/138 (Q1)

SEGUNDA PUBLICACIÓN:

- Título: Effect of cryopreservation and single layer centrifugation on canine sperm DNA fragmentation assessed by the sperm chromatin dispersion test

- Autores (p.o. de firma): M Urbano, J Dorado, I Ortiz, JM Morrell, S Demyda-Peyras, MJ Gálvez, L. Alcaraz, L Ramírez, M Hidalgo

- Revista (año,vol.,pág.): Animal Reproduction Science 143 (2013) 118-125

- Base de Datos Internacional o Nacional en las que está indexada: Journal of Citation Reports (JCR). 2013.

- Área temática en la Base de Datos de referencia: Agriculture, Dairy & Animal Science

- Índice de impacto de la revista en el año de publicación del Artículo: 1,581

-Lugar que ocupa/Nº de revistas del Área temática: 11/52 (Q1)

TERCERA PUBLICACIÓN:

- **Título**: Identification of sperm morphometric subpopulations in cooled-stored canine sperm and its relation with sperm DNA integrity

- **Autores (p.o. de firma):** M Urbano, I Ortiz, J Dorado, M Hidalgo

- **Revista (año,vol.,pág.):** Reproduction in Domestic Animal 52 (3): 468–476

- **Base de Datos Internacional o Nacional en las que está indexada:** Journal of Citation Reports (JCR). 2015.

- **Área temática en la Base de Datos de referencia:** Agriculture, Dairy & Animal Science

- **Índice de impacto de la revista en el año de publicación del Artículo:** 1,210

- **Lugar que ocupa/Nº de revistas del Área temática:** 19/58 (Q2)

CUARTA PUBLICACIÓN:

- **Título**: Comparison of DNA fragmentation of frozen-thawed epididymal canine sperm using SCSA and SCDt

- **Autores (p.o. de firma):** M Urbano, I Ortiz, J Dorado, JM Morrell, E Al-Essawe, A Johannisson, M Hidalgo

- **Revista (año,vol.,pág.):** Animal Reproduction Science (Submitted on April , 2017)

- **Base de Datos Internacional o Nacional en las que está indexada:** Journal of Citation Reports (JCR). 2015.

- **Área temática en la Base de Datos de referencia:** Agriculture, Dairy & Animal Science

- **Índice de impacto de la revista en el año de publicación del Artículo:** 1,377

-**Lugar que ocupa/Nº de revistas del Área temática:** 15/58 (Q2)

SCIENTIFIC PRODUCTION

PRODUCCIÓN CIENTÍFICA

PRODUCCIÓN CIENTÍFICA

Otras aportaciones científicas derivadas directamente de la Tesis Doctoral:

Otras Publicaciones en Revistas Indexadas en el JCR:

- M Urbano, J Dorado, I Ortiz, MJ Galvez, S Demyda-peyras, M Moreno, l Alcaraz, L Ramirez, F Quesada, C Gonzalez, JM Portero, D Acha, M Hidalgo (2013)."*Effect of a stressor on canine sperm DNA fragmentation using the SCD test"*. Reproduction, Fertility & Development 25 (1) 148-316. 39th Annual Conference of the IETS. Hannover (Alemania), 19-22 Enero, 2013.

- M Urbano, J Dorado, I Ortiz, M Galvez, L Alcaraz, L Ramirez, D Acha, S Demyda-Peyras, M Hidalgo (2013). "*Incubation of thawed canine sperm increases DNA fragmentation"*. Reproduction in Domestic Animal 48 (S1) 1–124. 17th Annual Conference of the European Society of Domestic Animals Reproduction Conference (ESDAR). Bolonia (Italia), 12-14 septiembre, 2013.

- M Urbano, J Dorado, I Ortiz, MJ Galvez, M Hidalgo (2014). "*Computer-assisted sperm head morphology analysis of chilled-stored dog spermatozoa"*. Reproduction in Domestic Animal 49 (S3) 1–102. 18th Annual Conference of the European Society for Domestic Animal Reproduction (ESDAR), Helsinki, Finland, 11–13 September 2014.