Instituto de Investigación
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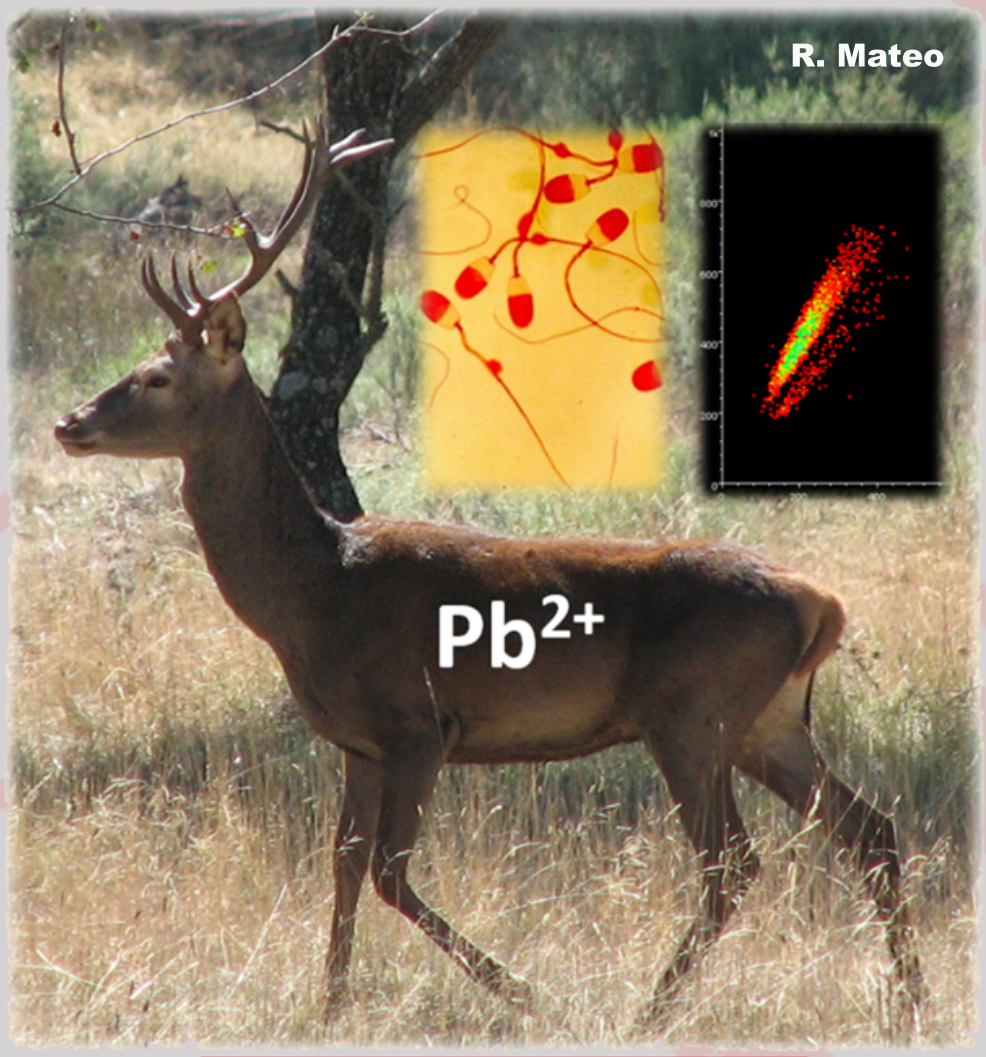
Efectos en la calidad espermática del ciervo de la exposición a plomo y otros metales pesados: aplicación de modelos de exposición in vitro con espermatozoides



Pilar Castellanos Expósito

Tesis Doctoral

Montaje de portada: Efecto del Pb sobre espermatozoides de ciervo ibérico en Valle de Alcudia-Sierra Madrona (Autor: Rafael Mateo Soria).

Efectos en la calidad espermática del ciervo de la exposición a plomo y otros metales pesados: aplicación a modelos de exposición *in vitro* con espermatozoides

Memoria presentada por

Pilar Castellanos Expósito

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UNIVERSIDAD DE CASTILLA-LA MANCHA. DEPARTAMENTO DE CIENCIA Y TECNOLOGÍA AGROFORESTAL Y GENÉTICA

Effects of lead and other heavy metals exposure on red deer sperm quality: application to *in vitro* exposure models with spermatozoa

Pilar Castellanos Expósito

PhD Thesis

A Manuel Castellanos y Mª Jesús Expósito,

In Memorian

A mis hijas Clara y Elisa,

Por ser SIEMPRE mi motor

A mi hermano Manolo,

Por ser como otro padre y

ejemplo de esfuerzo y

honestidad

"… Il y'a un devoir qui s'impose, aujourd'hui plus que jamais, aux hommes de science de penser leur discipline dans l'ensemble de la culture moderne pour l'enrichir non seulement de connaissances techniquement importantes, mais aussi des idées venues de leur science qu'ils peuvent croire humainement signifiantes. L'ingénuité même d'un regard neuf (celui de la science l'est toujours) peut parfois éclairer d'un jour nouveau d'anciens problèmes... "

Le hasard et la nécessité: essai sur la philosophie naturelle de la biologie moderne

"Todo ser vivo es también un fósil: Dentro de él todo el camino hasta la estructura microscópica de sus proteínas, conserva las huellas y hasta los estigmas de su ascendencia"

Jacques Monod (1910-1976). Premio Nobel de Fisiología y Medicina (1965)

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primera noche sola?"). Yo si la recuerdo. Perdona la lata que te he dado con el SPSS, aprendo poco y mal, ya me conoces.

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Se que estaríais orgullosos. Va por vosotros también.

Índice

Estructura

La organización de la presente tesis doctoral se ha realizado manteniendo el orden de contenidos del formato clásico y general de cualquier texto científico. Aparece en primer lugar una ***Introducción*** general en la que se exponen una serie de contenidos que a modo de soporte teórico ayuden a una mejor comprensión de los capítulos siguientes. En primer lugar se hace una aproximación relativa a la ubicuidad que presenta el plomo en el ambiente en base al uso que se ha hecho del mismo a lo largo de la historia y con especial atención al impacto derivado de las actividades de extracción minera por ser ésta la fuente de exposición al plomo y también a otros metales, que afecta a ungulados silvestres del área de Sierra Madrona-Valle de Alcudia, zona natural de la que se hace una breve descripción y donde se desarrolla el trabajo de campo de esta tesis. También se exponen los principales resultados de estudios anteriores realizados en estas zonas mineras relativos a los efectos tanto a nivel crónico como agudo de la exposición a metales del ciervo común expuesto a contaminación en base a lo concentración encontrada de diferentes metales en diferentes tejidos del mismo, como son el hígado, hueso, bazo y tejido reproductor. Por último se ha incidido en cómo la exposición al plomo presenta efectos adversos sobre reproducción masculina representando un factor importante que afecta a la disminución de la calidad espermática que puede comprometer la fertilidad del individuo. Para ello se ha hecho una valoración de los diferentes mecanismos fisiológicos y estructurales en los que este metal puede intervenir alterando la función reproductora, tanto de forma directa como mediada por la situación de estrés oxidativo celular que se origina por la presencia de

plomo tanto en el tejido reproductor como en el plasma seminal. En concreto, se ha valorado el efecto del plomo a diferentes niveles como son la espermatogénesis o a procesos postesticulares, alterando el funcionamiento neuroendocrino, la estructura y fisiología de la membrana del espermatozoide o la integridad en el empaquetamiento del ADN en la cromatina en el mismo. A continuación, en los ***Objetivos*** se recopilan aquellos que se abordan en particular en cada uno de los seis ***Capítulos*** de los que consta la parte central de este trabajo de tesis. Estos capítulos presentan la estructura clásica de un artículo científico y están escritos en inglés manteniendo el formato de la revista donde ya han sido publicados o van a ser enviados, y es por ello que no guardan una uniformidad formal entre ellos. En la ***Discusión*** general se exponen de forma cohesionada los resultados obtenidos en los estudios desarrollados en los diferentes capítulos para aportar una visión general sobre los efectos adversos observados y dar explicación a los mismos. Por último se enumeran las principales ***Conclusiones*** de este estudio.

Introducción General

1. Historia de la contaminación por plomo.

El plomo (Pb) uno de los tóxicos ambientales e industriales más difundidos en el planeta debido al uso con muy variados fines que se ha hecho de este metal por diferentes civilizaciones a lo largo de la historia, gracias a su facilidad para fundirse a temperatura moderada (327,4ºC) (Conesa y Schulin, 2010). El Pb es un metal pesado con masa atómica de 207,2 u que se encuentra de forma natural como elemento geoquímico de la corteza terrestre en una concentración media de 13 mg/kg y en raras ocasiones se encuentra como Pb elemental sino combinado de forma mayoritaria en sulfuros de Pb (PbS, galena) (Bjerregaard y Andersen, 2007). El Pb está constituido por cuatro isótopos estables (^{204}Pb, ^{206}Pb, ^{207}Pb, ^{208}Pb) y el estudio de sus proporciones ha permitido valorar las diferentes fuentes de contaminación antropogénica que han existido a lo largo de la historia.

El uso del Pb se remonta a 3000 años AC en Egipto (Gale y Stos-Gate, 1981) y restos de explotación minera de Pb a gran escala ya se daban en Grecia en el año 850 AC (Patterson, 1972). La mayor extracción y uso de Pb antes del periodo preindustrial corrió a cargo de la antigua Roma, ya que durante 400 años se llegaron a extraer hasta 60.000 Tm/año de este mineral destinado a recipientes de vidrio, cañerías, utensilios para cocinar, para concentrar el zumo de uva y para endulzar el vino al añadirlo en forma de acetato de Pb (Wittmers et al., 2002). Este último uso se extendió fundamentalmente entre la clase dirigente romana quedando patente su bioacumulación con el hallazgo de Pb en los huesos de restos arqueológicos pertenecientes a esta época.

El hecho de encontrar en los huesos de las tumbas de patricios una mayor concentración de este metal que el encontrado en las tumbas de plebeyos, y dado el efecto negativo del Pb en la fecundidad, hace pensar a algunos investigadores que un envenenamiento selectivo por Pb pudo contribuir a la caída del imperio (Nriagu, 1983b). La ingesta de Pb por la clase aristócrata se calcula pudo ser de 1 mg Pb/día (Wooley, 1984), muy superior a los niveles medios de 1.4 µg Pb/día que se estima en la actualidad para población de algunas zonas industrializadas y potencialmente expuestas al mismo (Melnyk et al., 2000).

A diferencia de lo que ocurre con otros elementos traza usados con fines económicos como el Cr, Fe, Mn y Ni, y cuya presencia en el suelo se ha asociado con aportaciones de origen geológico, la presencia del Pb en el suelo es en gran parte debida a la actividad humana como se ha demostrado utilizando isótopos estables del Pb (^{206}Pb/^{207}Pb) que permiten diferenciar el Pb natural y el antropogénico, y que permiten asegurar que en el último siglo el Pb ha alterado su ciclo natural de forma global como consecuencia de la actividad humana, haciéndolo aún si cabe más ubicuo (Patterson 1965). En concreto, cuando el Pb es de origen mineral hay una relación ^{206}Pb/^{207}Pb >1,35. Por el contrario, si es de origen atmosférico (asociado con la combustión de gasolina con Pb) la proporción de estos isótopos baja a 1,14-1,16, llegándose a registrar el valor más bajo de esta ratio entre 1930 y 1990 coincidiendo con el enorme desarrollo del tráfico rodado en zonas industrializadas. Esto es fundamentalmente debido a que en el siglo XX hubo un nuevo incremento de producción de este metal con motivo del uso de compuestos orgánicos de Pb como es el tetraetilo de Pb $(C_2H_5)_4Pb$ como antidetonante en la gasolina de automoción, que al combinarse con otros antidetonantes de la misma, se trasforma en bromuro de Pb, compuesto volátil que supone un nuevo aporte de Pb a la atmósfera aumentando con ello el impacto ambiental y el riesgo de

exposición a este metal. Con este aporte, las partículas de Pb dispersadas son inhaladas con el polvo contaminado y de esta forma se incrementan el riesgo potencial de este metal. Se calcula que con la combustión de un galón de gasolina (3,78 litros) se pueden emitir a la atmósfera hasta 4 g de óxido de Pb (Needleman, 1997). Esta exposición ambiental se ha mantenido hasta hace muy poco en bastantes países occidentales, como es el caso del nuestro, en el que según medidas llevadas a cabo en la década de los años 90 en áreas urbanas como Madrid había una cantidad de Pb en la atmósfera de 0,6 $\mu g/m^3$ de aire (Llop et al., 2013). Por otra parte, hasta un 65% del Pb atmosférico termina depositándose en el suelo (Kaste el al. 2003). Esto explica que la cantidad en el suelo de este metal actualmente es unas 1.000 veces superior a la de hace unos pocos cientos de años. Este metal depositado queda acumulado en la zona más superficial del suelo (Bindler, 2011), en sedimentos de cursos fluviales (Begy et al., 2009) o en turberas (Bao et al., 2010), pudiendo incorporarse progresivamente a la cadena trófica. Este último aspecto ha quedado evidenciado al analizar la proporción de isótopos de Pb en los anillos de crecimiento de árboles y poder observarse que entre un 10-30% del Pb incorporado en los vegetales se debe a la deposición atmosférica del mismo (Bindler et al., 2004).

La toxicidad del Pb en humanos es conocida desde la antigüedad. Dioscórides, médico de Nerón y cirujano en el ejército, la describió al afirmar que “el Pb hace que vague la mente”. Fue más tarde, en el siglo XVI cuando en los escritos médicos de Paracelso reapareció el envenenamiento por Pb descrito como “alteraciones de los trabajadores de las minas” caracterizados por fenómenos de parálisis, esplenomegalia, letargia o pérdida de dentición. Más tarde Ramazzini (1713) y Baker (1772) describieron el envenenamiento severo de Pb asociado a dolores abdominales, encefalopatías y parálisis. Ya en el siglo XIX, Laemuec explicó los mecanismos que

intervenían en la anemia originada por el Pb y Tanquerel, en el año 1839, hizo una primera y moderna descripción clínica del saturnismo en su famoso "Traité des maladies du plomb ou saturnines", en la que aparecía una exhaustiva descripción de 1.200 casos de envenenamiento por este metal (Legge, 1959). En 1987, el célebre toxicólogo Mateu Orfila escribió que, en base a las publicaciones hasta fecha, el plumbismo era uno principales temas tratados en la medicina hasta ese momento (Goyer 1996).

2. Contaminación por Pb debida a la actividad minera: el caso de Valle de Alcudia-Sierra Madrona.

Los amplios usos que se ha tenido del Pb explican la intensa actividad de extracción minera de Pb que se ha tenido que mantener a lo largo de la historia para proporcionar a la población las cantidades suficientes de este metal. Esto ha producido un impacto ambiental en muchas áreas de Europa que no se limita a las prácticas de extracción poco eficaces sin medios para prevenir el daño ambiental producido, sino también al abandono de gran parte de las explotaciones mineras sin aplicar ninguna medida de remediación (p.e. fitorremediación) de las áreas mineralizadas, continuando así la dispersión de la contaminación (Dybowska et al., 2006). Sin embargo, en las últimas décadas, y para evitar la dispersión de metales residuales de la contaminación minera, muchos estudios se han encaminado a identificar plantas nativas que permitan una rápida cobertura y que puedan desempeñar una fitoestabilización del metal (Sun et al., 2005).

En lo que a nuestro país se refiere, durante los siglos XIX y XX la extracción y fundición de los sulfuros de Pb para la obtención del mismo fue una de las actividades

con mayor impacto económico, siendo la comarca minera del Valle de Alcudia y Sierra Madrona la que se encontraba a la cabeza de producción durante este periodo con 130.000 toneladas métricas anuales (Pérez de Percebal Verde y Sánchez-Picón, 2001). Para comprender la repercusión sobre el medio ambiente de esta actividad minera hay que tener en cuenta tanto las características del sustrato geológico como la historia de dicha actividad. A nivel geológico, el suroeste de la península Ibérica se encuadra en un cinturón de pirita de 250 km de longitud y que constituye a su vez la mayor extensión periférica de la orogenia hercínica europea. En concreto y en lo que se refiere a la región del Valle de Alcudia-Sierra Madrona, a las que pertenecen las muestras analizadas en el trabajo de campo de esta tesis, el sustrato geológico corresponde al conocido como relieve apalachense formado por una sucesión de sierras y depresiones con montañas de media altura y formaciones volcánicas. En este relieve formado durante el Precámbrico Superior y Paleozoico Inferior y Medio, el basamento antiguo presenta un predominio de cuarcitas, areniscas y pizarras con metamorfismo de bajo grado (García Rayego, 1999), entre las que aparecen filones de relleno de Pb, zinc, plata, hulla, y a lo que además se añade los yacimientos de cinabrio de Almadén (Palero-Fernández, 2000). Por otra parte, la oxidación progresiva que han ido sufriendo los sulfuros metálicos presentes en esta comarca es un aspecto determinante para explicar la movilización algunos metales, ya que se sabe que los procesos de oxidación que tienen lugar en los depósitos metalíferos provocan la acidificación de suelos y aguas próximos, y con ello se incrementan tanto la solubilidad como la biodisponibilidad de ciertos metales como Pb, Ni y Zn, aumentando con ello su riesgo potencial (Prica et al., 2010). A estas particularidades geológicas se añade el hecho de que en la franja suroeste de la provincia de Ciudad Real, ha habido un aporte casi continuado de Pb y otros metales a suelos y aguas como consecuencia de la importante actividad minera de

algunas zonas del Valle de Alcudia y Sierra Madrona, en cuyo distrito minero se distinguen dos vertientes: la del cinabrio de Almadén-Almadenejos al oeste (Millán et al., 2011) y la de galena argentífera en el centro y mitad oriental (Palero-Fernández y Martín-Izard, 2005).

El origen de la minería en la provincia de Ciudad Real se remonta a la época prerromana con la extracción de cinabrio en el siglo IV a.C., y se acrecentó durante la expansión romana en nuestra península con la extracción de galena argentífera de forma continua en Mina Diógenes y San Quintín. Tras el freno en la época visigoda, los árabes revitalizaron la minería de la provincia, a lo que se le añadió su impulso después del descubrimiento de América, en base a factores que la favorecieron tales como los conflictos bélicos (cañones, balas), el incipiente desarrollo urbano (hierro para la clavazón) y la posterior colonización del territorio con aumento demográfico (aperos de labranza) (Gómez-Vozmediano, 2000). A esta actividad minera se le sumó en la Edad Media la revitalización de la extracción de mercurio en Almadén y aunque esta actividad disminuyó durante los siglos XVII y XVIII, se recuperó en la segunda mitad del XIX, siendo entonces esta zona la mayor productora de Pb del país (Hevia, 2003). La actividad minera ha condicionado el desarrollo de esta comarca al ser el motor económico de todo el Valle de Alcudia hasta el descenso de su actividad a partir de 1930, que finaliza en 1988 cuando se cierra la mina de San Quintín. Como resultado de toda esta actividad en la provincia se contabilizan actualmente unas 484 minas antiguas con venas de Pb y zinc (Palero-Fernández y Martín-Izard, 2005). Estas explotaciones abandonadas siguen provocando problemas de diseminación de metales a lo largo de 2.500 km^2. En algunas localidades mineras estos vestigios de actividad han sido considerados como monumentos culturales o patrimonio de interés para los visitantes (Álvarez y Piegia, 2010). El deterioro ambiental de las antiguas zonas mineras supone

un mayor impacto, si cabe, al ser ésta una zona ambientalmente protegida, ya que las 226.000 ha del Valle de Alcudia y Sierra Madrona han sido declarados Parque Natural (Ley 6/2011, 10 de marzo) y cuenta con diferentes figuras de protección ambiental como son Lugar de Importancia Comunitaria (LIC: ES 4220014), Zona de Especial Protección para las Aves (ZEPA: ES0000090), Área de Importancia para las Aves (IBA: 207). Esta zona minera se extiende hacia las provincias limítrofes de Jaén, Córdoba y Badajoz, por lo que se pueden dar problemáticas similares a la de Ciudad Real (Santiago et al. 1998; Millán et al., 2009; Sevillano-Morales et al., 2011).

Además de este impacto ambiental, esta situación provoca una grave riesgo para la fauna silvestre de la zona, ya que existe una transferencia de metales a la cadena trófica a través de los vegetales debido a que las partículas metálicas sufren una dispersión aérea hasta su deposición en la vegetación de cubierta, para ser finalmente ingeridas por los herbívoros silvestres y domésticos (Reglero et al., 2008; Rodríguez-Estival 2012).

El riesgo de transferencia de metales a la biota de forma espontánea en zonas mineras abandonadas se incrementa cuando los suelos afectados son colonizados por especies nativas que actúan de bioacumuladoras de los mismos, ya que según algunos estudios se han llegado a medir concentraciones de 10 mg/kg de Cu, 200 mg/kg de Pb y 500 mg/kg de Zn en algunas especies (Conesa y Shulin, 2010). De esta forma se acentúa el riesgo de que estos metales sean transferidos a mamíferos herbívoros (Madejón et al., 2002), tal y como se ha comprobado en ciervos del Valle de Alcudia - Sierra Morena, donde los niveles de Pb en sedimentos de arroyos se encuentran entre 44-1.481 μg/g, (Reglero et al., 2008), y por lo tanto superan los niveles medio en Europa (Tabla 1). Por ello, las consecuencias negativas sobre el medio ambiente como resultado de la potente actividad extractiva persisten en la actualidad en diversas zonas del Valle de Alcudia y

Sierra Madrona (Fig. 1), con niveles medios de Pb de 15.175 mg/kg en escombreras, 2.209 mg/kg en suelos circundantes y 1.785 mg/kg en los sedimentos de los cursos de agua próximos (Higueras et al., 2011), todos ellos valores por encima de los valores medios registrados en Europa (Tabla 1).

Tabla 1: Niveles medios de Pb en suelos y aguas de Europa en µg/g (Salminen 2005)

SUSTRATO	**Mínimo**	**Medio**	**Máximo**
Suelo superficial	5.32	32.6	970
Humus	0.80	56.8	536
Agua	<0.005	0.224	10.6
Sedimentos móviles	<1.0	38.6	5760

Fig. 1. *Fotografía aérea de un grupo de minas abandonadas del Valle de Alcudia.*

Los niveles de Pb en el suelo en el área minera de Valle de Alcudia-Sierra Madrona causan una acumulación del mismo en diferentes especies vegetales, encontrándose en mayor concentración en la encina (*Quercus ilex*) y la jara pringosa (*Cistus ladanifer*), llegando a medirse valores de hasta 1,000 μg/g peso seco (p.s.) de Pb (Reglero et al., 2008). En general, la mayor parte del Pb presente en las plantas queda retenido en las raíces (Wang et al., 2012), por lo que resulta más expuesto al Pb el jabalí, que se alimenta en buena parte de raíces, que el ciervo, que se alimenta principalmente de hojas (Rodríguez-Estival et al., 2011).

3. Efectos del Pb en los animales de la zona minera de Valle de Alcudia-Sierra Madrona.

En estudios anteriores realizados en las zonas mineras del Valle de Alcudia y Sierra Madrona se ha encontrado una clara relación entre las cantidades de Pb encontradas en sedimentos y suelo, en plantas y en diferentes tejidos de ungulados como el ciervo rojo (*Cervus elaphus*) y el jabalí (*Sus scrofa*). Una de las zonas mineras en las que se han encontrando los valores más elevados de Pb y otros metales ha sido en los alrededores de la antigua Mina de Horcajo (Reglero et al., 2008). En los sedimentos de esta zona se midieron concentraciones de Pb de 1.481 μg/g p.s., comparables a las encontradas en otras zonas con minería de este metal (Liu et al., 2005; Deng et al., 2006), y de arsénico de 1.880 μg/g p.s, que son niveles alrededor del doble de los encontrados en las zonas más contaminadas del vertido minero de Aznalcóllar y dentro del rango de concentración asociado con trastornos en la biota (Taggart et al., 2005). Esta concentración tan elevada de As en suelos lleva a encontrarlo en cursos de agua cercanas a estas minas en concentraciones cinco veces superiores a las aceptadas como

medida estándar de este metal para consumo humano (WHO, 2006). Igualmente en la Mina de Horcajo, la concentración de Cu en suelo fue de 66 μg/g p.s., similar a la encontrada en áreas mineras del NE de Portugal (Freitas et al., 2004b), y los niveles de Se en suelos y sedimentos fueron superiores a 0,2 μg/g p.s. que son los considerados como normales (Kabata-Pendias y Pendias, 1992), si bien los niveles variaron según la zona minera. Por otra parte, se encontró en estas áreas una concentración de Pb más alta que en zonas control en un total de 13 especies vegetales muestreadas. Sin embargo, y de cara a evaluar el grado de exposición a metales en los herbívoros de las áreas mineras, se sabe que en términos de transferencia a la biota, la ingestión de metales que se encuentran en las partículas depositadas en la superficie de las plantas puede representar un aporte más importante que las cantidades de metal primeramente absorbidas por las raíces y trasferidas más tarde a la parte aérea (Madejón et al., 2002). En el caso concreto de ungulados habría que añadir a esta ingestión accidental de metales a través de partículas de suelo contaminadas y depositadas sobre hojas y frutos (Beyer et al., 1994), la ingestión intencionada de pequeñas fracciones de suelo, conocida como geofagia (Klaus y Schmid, 1998), y que en ocasiones se utiliza como recurso para suplementar la carencia de nutrientes (Mincher et al., 2008). De cualquier modo, esta transferencia de Pb y otros metales desde el suelo a la biota ha ocasionado que en el ciervo rojo de las áreas mineras del Valle de Alcudia y Sierra Madrona se hayan encontrado concentraciones en el hígado en p.s de 0,805 μg/g de Pb, 0,554 μg/g de Cd, 0,327 μg/g de Se y 0,061 μg/g de As, y siempre superiores a las encontradas en zonas control (Reglero et al., 2008). En el hueso de estos animales, la concentración de Pb fue más elevada que en el hígado (Reglero et al., 2009a), lo que muestra el efecto crónico de la contaminación por metales que sufre la fauna de estas zonas. Teniendo en cuenta que en el hueso, el Pb puede llegar a representar más del 90% del contenido total de este

metal en el organismo (Ma, 1996), se puede explicar la modificación tanto de la composición de este tejido como de los procesos de mineralización y renovación ósea por exposición a este metal que, como muestran algunos estudios (Gangoso et al., 2009), reduce el depósito de minerales en el hueso. Este efecto también se ha observado en el ciervo y jabalí de estas zonas mineras con mayor presencia de Pb en hígado y hueso unida a un menor contenido de carbonato mineral en este tejido (Rodríguez-Estival et al., 2013), y una reducción en el hígado de ésteres de retinol, acompañada de un aumento de retinol libre (Rodríguez-Estival et al., 2011; Rodríguez- Estival et al., 2013). La disminución en la vitamina A que se ha encontrado en estos animales, hace pensar que los cambios en la composición y mineralización del hueso observada está probablemente mediada por el agotamiento de esta vitamina en la fauna expuesta a contaminación por Pb al actuar ésta como defensa antioxidante bajo condiciones de estrés. Por otra parte, en jabalíes de zonas mineras también se ha encontrado una relación negativa entre los niveles de α- tocoferol en el hígado y la concentración de Pb en el hueso, que podría ser indicador de algunos efectos a largo plazo del Pb en los niveles de antioxidantes de estos animales expuestos a este metal (Rodríguez-Estival et al., 2011). Esta alteración en la defensa antioxidante no solo se ha encontrado en hígado, sino que en el caso ciervos machos adultos, también se midieron niveles inferiores de palmitato de retinol en el testículo. Esto último puede estar relacionado con el aumento del uso interno de esta vitamina con la finalidad de tratar de mantener la integridad y funcionalidad del tejido reproductivo (Rodriguez-Estival et al., 2011), ya que como se ha demostrado en otros estudios estas vitaminas tienen un papel importante en el mantenimiento de buenos parámetros de calidad espermática (Kefer et al., 2009).

Comparando el efecto del Pb en las dos especies, en caso del jabalí las concentraciones de este metal tanto en el hígado como en el hueso son superiores que

las encontradas en el ciervo, probablemente condicionada a una mayor ingestión por parte del jabalí de suelo, bulbos y raíces de zonas contaminadas (Taggart et al., 2009). Además, de forma más marcada que en el ciervo, en el caso del jabalí hay correlación entre las cantidades de Pb en el hígado y en el hueso (Reglero et al., 2009a), lo que sugiere que en esta especie hay una mayor deposición de Pb en este último tejido. Por otra parte, en los dos ungulados se encontró un mayor nivel de Pb en el bazo en relación a animales de las zonas control (Rodríguez-Estival et al., 2013), unido a una reducción de los niveles de glutatión en el bazo y cambios en la actividad de la enzima antioxidante glutatión peroxidasa (GPx). Igualmente, la presencia de Pb en este órgano linfoide produjo cambios en el sistema inmune de estos animales hacia diferentes vías de respuesta que, sin embargo, no aparecieron directamente relacionados con parámetros de estrés oxidativo. En concreto en el caso del ciervo hubo una inducción de interleucina IL-4 en los machos de la zona minera, mientras que en jabalí se vio aumentada la expresión de genes del gamma interferón (IFN- γ) (Rodríguez-Estival et al., 2013).

La concentración hepática de Pb en ciervos de Valle de Alcudia y Sierra Madrona está muy por debajo de la asociada con signos clínicos de envenenamiento por Pb (30 μg/g p.s o 10 μg/g en p.f., Ma, 2011; Frøslie et al., 2001), y es similar a los niveles medios de Pb en ciervo común de diferentes zonas de Europa (0,03- 2,5 μg/g p.f; Frøslie et al., 2001), o incluso son inferiores a los encontrados en corzo (*Capreolus capreolus*) de otras áreas mineras de Europa (0,71 μg/g p.f., Pokorny y Ribarï-Lasnik, 2000) o a ciervos comunes de Sierra Morena (0,57 μg/g p.f., Santiago et al., 1998). Sin embargo, la exposición al Pb y otros metales pesados en esta zona minera ha provocado efectos subletales como el descenso en los niveles de antioxidantes exógenos como las vitaminas A y E encontrado, que reflejan una alteración en el balance prooxidantes-

antioxidantes producido por la exposición a este metal (Rodriguez-Estival et al., 2011). Así por ejemplo, se ha medido un significativo 39% de disminución del glutatión (GSH) hepático ligado a un descenso en los niveles de los ácido graso poliinsaturados (PUFAs) n-3 y en concreto del ácido docosahexaenoico (DHA:22:6n-3), ácido graso que por tener seis dobles enlaces es especialmente vulnerable al ataque oxidativo y que se vio disminuido en un 16% (Reglero et al., 2009a). Sin embargo, en algunos casos el descenso del GSH no fue acompañado con la disminución de actividad de otras enzimas antioxidantes, como la glutatión peroxidasa (GPx) o la superóxido dismutasa (SOD), lo que puede deberse a la función del GSH en la excreción activa por la bilis del Pb acumulado en el hígado (Klaassen y Shoeman, 1974), como consecuencia de la unión de este metal al grupo tiol (-SH) del GSH (Donaldson y Knowles, 1993). Pese a lo anterior, en otros ciervos analizados correspondientes a años diferentes, pero también con un nivel elevado de Pb hepático, el descenso en los niveles de GSH estuvo acompañado por la disminución del α- tocoferol y del la GPx, si bien ésto no ocurrió en el jabalí, en el que la mayor concentración de Pb en hígado estuvo relacionada además de con mayor nivel de Se y con un aumento en la actividad de la GPx. Todo indica a que en las áreas mineras estudiadas, el efecto negativo de este metal sobre antioxidantes endógenos y exógenos es superior en el caso de ciervo (Rodríguez-Estival et al., 2011).

4. Efecto del Pb en la función reproductora masculina.

En el momento actual hay una preocupación por el descenso de la fertilidad humana de la que se conoce que entre un 30-40% de los casos tiene como causa la infertilidad masculina debido al descenso global que está sufriendo la calidad espermática a causa de diferentes factores ambientales ((Marzéc-Wróblewska et al.,

2012; Deonandan y Jaleal, 2012). Esto ha llevado no solo a profundizar sobre las causas de este descenso, sino también a completar la evaluación clásica de los parámetros de calidad espermática como son la motilidad y la calidad de movimiento, la integridad del acrosoma del espermatozoide y la funcionalidad de la membrana, con otras medidas que permitan explicar algunos tipos de infertilidad idiopática que no se podría justificar en base a los parámetros convencionales de fertilidad (Aitken et al., 1984a; Bonde et al., 1998). Estos nuevos parámetros incluyen la evaluación de los procesos de estrés oxidativo celular que es responsable, entre otros, de la alteración de la estructura y funcionalidad de la membrana del espermatozoide y de daños oxidativos en su ADN. Esta fragmentación del ADN limita no solo la capacidad fecundante de la célula (Bungum, 2012, Sakkas y Álvarez, 2010; Zini y Sigman 2009), sino que puede afectar también a las células de la línea germinal y alterar el proceso meiótico de la espermatogénesis (Nagy et al., 2013). Por otra parte, el estudio de estos mecanismos bioquímicos y moleculares que afectan a la fertilidad ha ido aportando a su vez más datos sobre la confluencia de causas que están detrás de esta disminución de la calidad espermática (Fig. 2).

Se conoce el efecto negativo del Pb y otros metales sobre la calidad del espermatozoide en mamíferos (Marzec-Wróblewska et al., 2012) y la relación que guarda la presencia de este metal en sangre o semen con la infertilidad masculina (Li et al., 2012). Así por ejemplo, se ha visto que en hombres infértiles no expuestos ocupacionalmente al Pb y que presentaban oligospermia (nº escaso de espermatozoides), astenospermia (poca motilidad) y azoospermia (ausencia de espermatozoides), la concentración de Pb en sangre era de 125 ng/ml, mientras que esta concentración en el caso de varones fértiles presentaba valores medios de 60 ng/ml

(Pant et al., 2003). Por otra parte, este efecto también se ha evidenciado en estudios in vitro (Ghaffari y Motlagh, 2011).

El efecto tóxico del Pb para la función reproductora tiene un carácter multifactorial, ya que este metal puede actuar a diferentes niveles. El riesgo de exposición al Pb y otros metales pesados se inicia ya durante el desarrollo embrionario como consecuencia de la interacción de estos metales con la barrera placentaria (Caserta et al., 2013) siendo responsable no solo de abortos, retraso en el crecimiento intrauterino y malformaciones fetales (Wirth y Mijal, 2010; Apostoli y Catalani, 2011), sino también de serios trastornos endocrinos que se manifiestan desde las primeras etapas del desarrollo, y que pueden estar detrás de la maduración reproductiva retardada por una alteración del proceso de espermatogénesis (Tabla 2). De esta manera, se ha encontrado en ratones de experimentación nacidos tras una exposición gestacional a Pb que se produce un retraso de la pubertad, además de un descenso en el peso de los testículos y un aumento del peso de la vesícula seminal (Sant'Ana et al., 2001).

Por otra parte, tras la espermatogénesis la toxicidad del Pb puede seguir actuando sobre los procesos post-testiculares de maduración y capacitación del espermatozoide. Durante estos procesos tanto la hipermotilidad que ha de adquirir la célula, como la reacción del acrosoma en la que se liberan las enzimas necesarias para la fertilización del óvulo tienen lugar unos complejos e interrelacionados cambios en la estructura y fisiología de la membrana del espermatozoide que además de ser calcio-dependientes, regulan la concentración de este ión a nivel intracelular de la que dependen estos procesos (Lishko et al., 2010; Visconti et al., 2011). El efecto tóxico que ejerce el Pb a este nivel puede deberse, por una parte, a la conocida competencia del Pb^{2+} con el ion calcio (Ca^{2+}), que le lleva a interferir con él en muchos mecanismos fisiológicos controlados por el mismo (Chen et al., 2009), y por otra parte, a los cambios

en la permeabilidad en la membrana del espermatozoide muy rica en ácidos grasos poliinsaturados fácilmente oxidables (Del Barco-Trillo y Roldán 2013).

Tabla 2: Lugares de acción en el sistema reproductor masculino del exceso de metales. A: acción celular directa; B: alguna acción directa y por mediador; C: deficiencia causa toxicidad (Thomas 1995a).

Metal	Hipot.-hipóf.	C.Germinal	C.Leydig	C.Sertoli	Mecanismos
Cd	Ninguno	B	B	B	Hipoxia/isquemia
Zn	Ninguno	C	B	C	Efecto por deficiencia
Pb	Supresión de FSH y LH	A	A	B	Toxicidad endo y paracrina

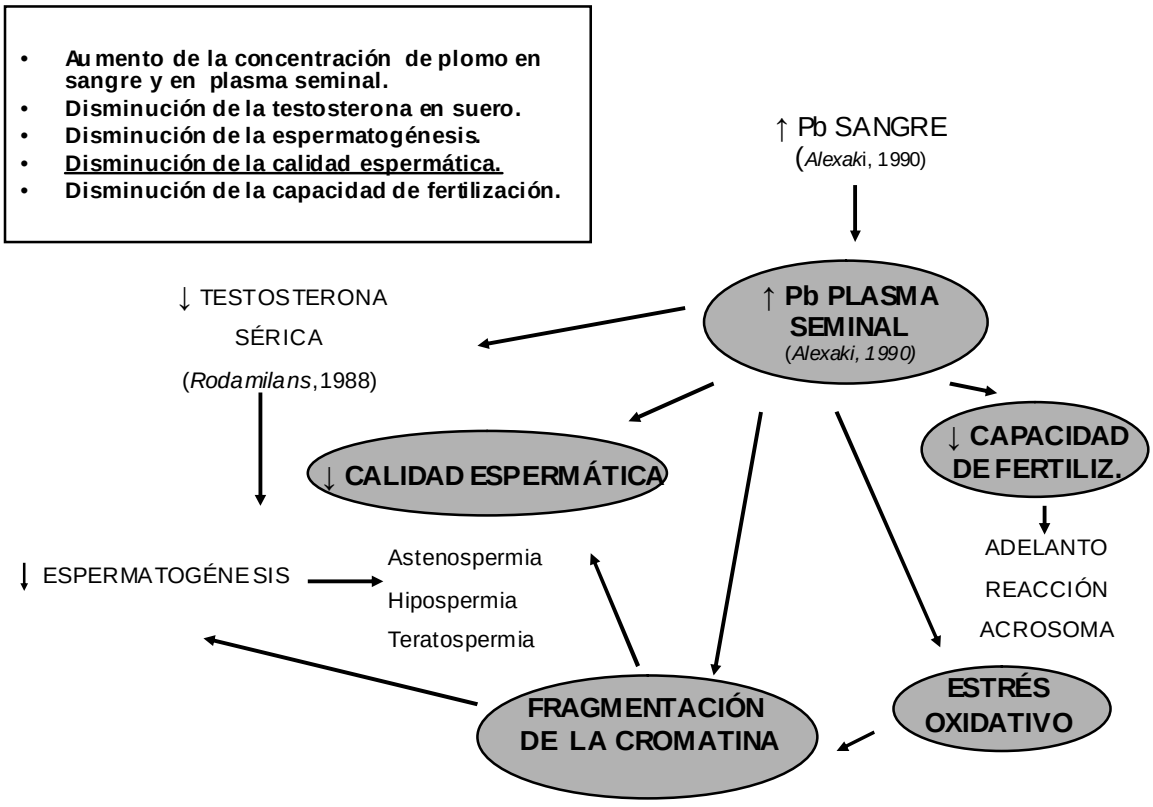

Figura 2. *Efectos del Pb en plasma y semen sobre la reproducción*

4.1. Efecto del Pb en la espermatogénesis a través del eje hipófisis-hipotálamo-testículo y por alteraciones histopatológicas

La función gametogénica y secretora de los testículos depende de un complejo mecanismo hormonal en el que intervienen de forma coordinada tres glándulas endocrinas, el hipotálamo (estructura neurosecretora), la hipófisis o glándula pituitaria concretamente el lóbulo anterior de la misma (adenohipófisis) y los testículos, y que se conoce como eje glándula pituitaria-hipotálamo-testículo. El proceso se inicia cuando las neuronas del hipotálamo neuroendocrino segregan factores específicos bien de la liberación o de inhibición de la liberación de hormonas al sistema sanguíneo que son conducidos a la adenohipófisis. Allí actúan sobre una subpoblación de células gonadotropas basófilas de la adenohipófisis que a su vez segregan dos hormonas gonadotropinas de carácter glicoprotéico, la hormona folículo estimulante (FSH) y de la hormona luteinizante (LH), también conocida como ICSH (hormona estimulante de las células intersticiales). Por otra parte, la secreción de FSH y LH por la adenohipófosis está también regulada por hormonas gonadales esteroides. Ambas hormonas hipofisiarias son trasportadas por la sangre hacia el testículo para actuar sobre diferentes células diana. Por una parte, la LH actúa sobre las células de Leydig, que están situadas en el espacio intersticial de los túbulos seminíferos y estrechamente asociadas con los vasos sanguíneos de este espacio, para estimular la síntesis de testosterona por estas células (Herbert et al., 1995). La FSH actuando sobre las células de Sertoli localizadas en la pared de los túbulos seminíferos se encarga de estimular el proceso de espermatogénesis (Heckert y Griswold, 1993). Las estrechas uniones entre las células de Sertoli forman la barrera sangre-testículo que divide al epitelio seminífero en dos compartimentos: el compartimento basal y el compartimento adluminal que contienen

células en diferente grado de diferenciación asociadas en bandas membranosas que contienen una generación celular formada a partir de una espermatogonia inicial. Durante este proceso de duración variable, las espermatogonias sufren múltiples mitosis y se transforman en espermatocitos I (primarios), aún diploides, que posteriormente por división meiótica formarán espermatocitos II (secundarios), células haploides que se irán diferenciando primero en espermátidas redondeadas y tras la eliminación de gran parte del citoplasma en espermatozoides aún inmaduros, de modo que en el epitelio germinal de los túbulos seminíferos coexisten células en diferentes estadios de diferenciación (Guyton, 1995; Fig. 3).

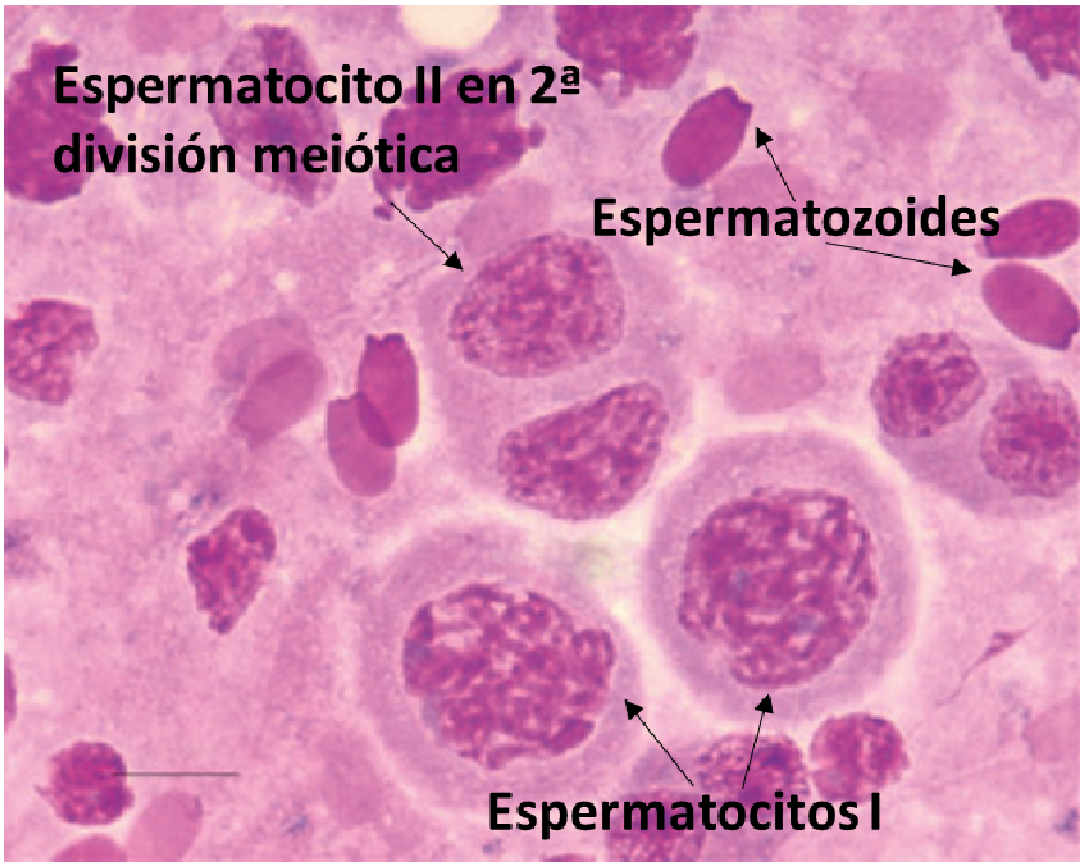

Figura 3. *Microfotografía de células germinales de ciervo común (Pintus et al., 2014).*

Las células de Sertoli, además de soporte para la línea germinal, son las que controlan el tránsito de hormonas y nutrientes necesarios para el proceso de diferenciación de espermatogonias. Además, las células de Sertoli ayudan a la regulación de todo el proceso al segregar pequeñas cantidades de estrógenos, de una hormona llamada inhibina y de la proteína ABP (proteína fijadora de andrógenos) que ayuda a modular el proceso de espermatogénesis y que son usadas como medida de integridad funcional tanto del epitelio como de las células de Sertoli (Mann y Lutwak-Mann, 1981). De ahí la reconocida importancia de las células de Sertoli para una normal espermatogénesis (Foster, 1992), y explica la alteración de este proceso que se produce por la presencia de muchos tóxicos que actúan directamente sobre estas células más que sobre las células germinales. La duración del ciclo de este epitelio es muy variable según las especies, y si bien para que se complete la espermatogénesis en los mamíferos se requiere entre 4,3 y 4,7 ciclos, que unido al tránsito por el epidídimo llega a ser entre 30-75 días en mamíferos el tiempo que requiere la formación de espermatozoides maduros (Rusell et al., 1993).

Por último hay que añadir que un defecto en la función testicular (en la producción de espermatozoides o testosterona) tenderá a incrementar los niveles de FSH y LH por la falta de retroalimentación negativa de este sistema y afectando a la correcta espermatogénesis (Thomas y Thomas, 1994). Por todo ello, la presencia de tóxicos que interfieran sobre estructuras o procesos a diferentes niveles del eje glándula pituitaria-hipotálamo-testículo puede también alterar este proceso de espermatogénesis (Fig. 4).

La muy conocida neurotoxicidad del Pb y su efecto sobre el eje hipotálamo-adenohipofisis-gonada es el factor que explicaría alguna de las alteraciones endocrinas que aparecen con la exposición a este metal, tanto en humanos como en animales, asociadas tanto a una deficiente espermatogénesis como a una disminución de la calidad

espermática que podrían comprometer la capacidad reproductora masculina (Apostoli et al., 1998; Telisman, et al., 2000, Mason et al, 2014).

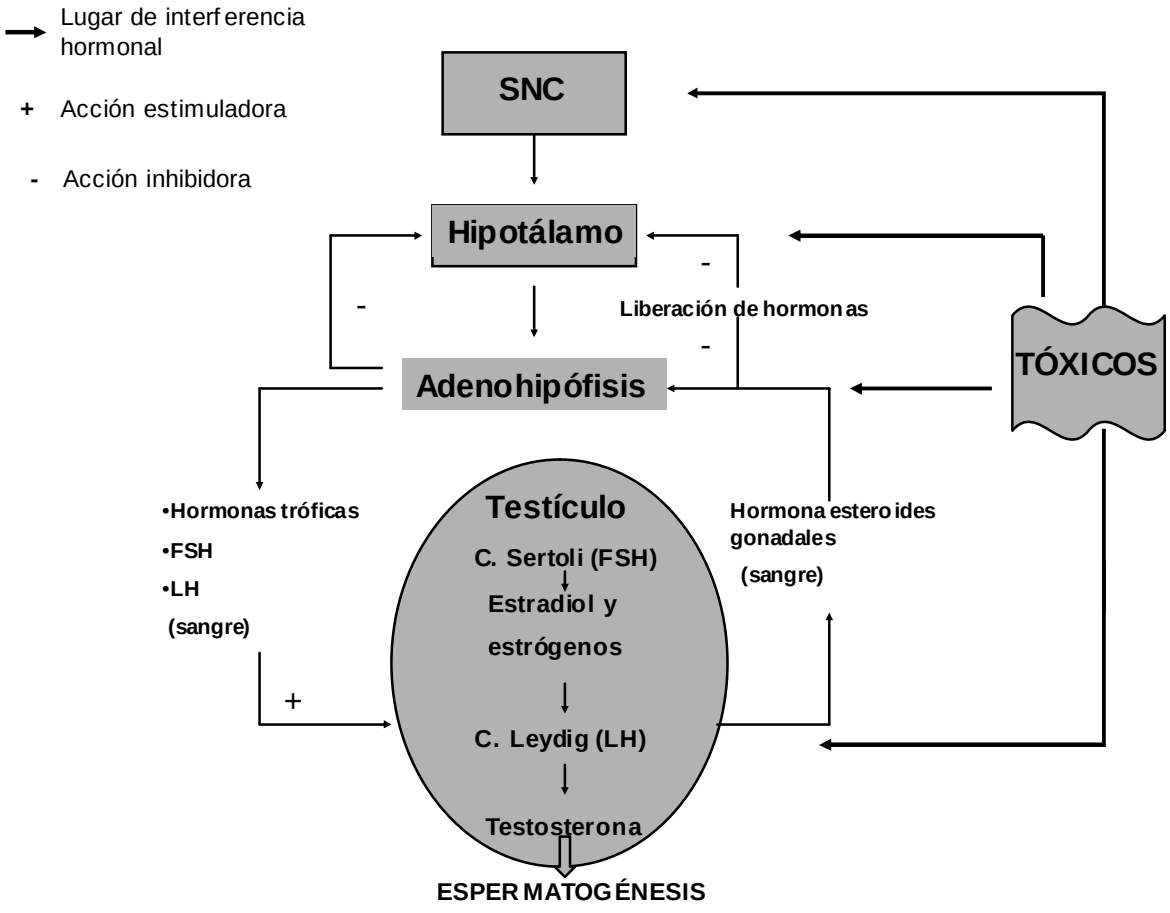

Figura 4. *Efecto de agentes tóxicos sobre el funcionamiento del eje hipotálamo-adenohipófisis -gónada y su efecto sobre la espermatogénesis (adaptado de Thomas et al., 1982).*

Como se ha comentado, las estructuras neuronales de este eje ya se ven afectadas durante el periodo gestacional por la exposición al Pb, ya que en este momento existe una importante proliferación neuronal. Así, en estudios en ratas expuestas a Pb durante la gestación se han observado alteraciones anatómicas como son

la disminución en un tercio de su volumen del núcleo sexualmente dimórfico del área preóptica del hipotálamo que provoca en la edad adulta una liberación irregular de las hormonas folicular (FSH) y luteínica (LH) (Mc Givern et al., 1991), y de alteraciones en la diferenciación fetal del eje hipófisis-hipotálamo-pituitaria-gonadal ligadas también a una reducción de la capacidad reproductora en el individuo adulto (Mc Givern et al., 1991). Sokol et al., (1987) sugieren que estos cambios anatómicos y neuroquímicos del cerebro son los que provocan un marcado descenso de los niveles de testosterona en plasma de hombres que han estado expuestos a este metal. Por otra parte, algunos autores apuntan a que el hipotálamo es el primer y principal lugar de acción del Pb, actuando sobre la prominencia media del mismo y así provocando una alteración del funcionamiento del eje hipotálamo-adenohipófisis-testículo (Stumpf et al., 1980). Esto explicaría la inhibición de la función testicular en animales adultos con niveles de Pb en plasma de inferiores a 40 μg/dl y que fueron expuestos a este metal en diferentes momentos del desarrollo en los que ha habido una sensible disminución de los niveles de testosterona plasmática e intratesticular y de la hormona FSH, siendo el descenso de testosterona más drástico cuando hubo exposición prenatal a este metal, sugiriendo que también afecta a los patrones de liberación de la hormona de crecimiento hipofisaria (Ronis et al., 1996).

Otro de los mecanismos de toxicidad del Pb sobre la espermatogénesis puede estar ligado a algunas modificaciones histológicas en los túbulos seminíferos que se producen por éste y otros metales pesados unido al desequilibrio antioxidante en el tejido reproductor (Al-Attar, 2011). Así, en animales de experimentación expuestos a este metal en los que la espermatogénesis queda disminuida o alterada se han encontrado daños en la membrana basal del epitelio germinativo de los túbulos seminíferos que han provocado una desorganización del mismo, una vacuolización

generalizada de las células llegando a quedar afectada la ultraestructura de las células de Sertoli (Bizarro et al., 2003) e incluso una disminución en el número de células germinales que lleva a encontrar unos túbulos seminíferos casi vacíos en aquellos casos en los que la dosis de Pb está entre 50-200 mg/Kg (Batra et al., 2001). Esta vacuolización en el epitelio germinativo también lo observaron Murthy et al. (1991) con concentraciones de 200 ng/ml de Pb en sangre. En este caso también se encontró en las células de Sertoli un incremento en el número y tamaño de los lisosomas que podría comprometer no solo el soporte metabólico que estas células aportan a la línea germinal, sino alterar la fijación de la FSH al actuar las células de Sertoli como células diana para ésta.

4.2. Efectos del Pb en los procesos post-testiculares

El producto final de la gametogénesis testicular son espermatozoides inmaduros que se desplazan por el fluido producido por los túbulos seminíferos dentro de un sistema de conductos llamados *rete testis* hacía el tortuoso conducto del epidídimo que cuenta con tres tramos (Amann, 1987), siendo en la cabeza y cuerpo donde maduran los espermatozoides y continúan su tránsito durante 1,8-5,4 días en el caso de mamíferos hasta la cola donde quedan almacenados (Rusell et al., 1993). La secreción de los órganos accesorios a las vías genitales (próstata, vesícula seminal, glándulas de Cowper y glándulas de Littre) es la responsable de mantener la homeostasis del plasma seminal, que es isotónico y neutro en muchas especies y cuenta con fructosa y sorbitol como fuente de energía, para permitir la capacitación del espermatozoide que se inicia en su avance por los conductos reproductores. En este momento, la presencia de tóxicos ambientales como el Pb pueden provocar un aumento de especies reactivas del oxígeno

(ERO) que alteran el sistema de antioxidantes naturales endógenos presente en el plasma seminal (O'Flaherty et al., 1997; Potts et al., 2000). Entre estos antioxidantes está el Zn segregado por la próstata (Kelleher et al., 2011), que tiene especial relevancia para asegurar una buena calidad espermática (Colagar et al., 2009), y el ácido cítrico procedente de la vesícula seminal (Lewis et al., 1997). Estos procesos de capacitación requieren un nivel concreto de oxidación y la presencia de pequeñas cantidades de ERO en el plasma seminal para regular una correcta función espermática ((Noblanc et al., 2012; Chen et al., 2013). No obstante, el espermatozoide es extremadamente sensible al daño oxidativo (Aitken et al., 2012), de modo que el equilibrio que se tiene que lograr entre oxidación "beneficiosa" y un exceso de la misma, puede alterarse fácilmente por la presencia de Pb en el plasma seminal. Este efecto se ha mostrado en diversos estudios realizados en hombres con exposición laboral en los que se han producido daños estructurales en la célula ya formada como consecuencia de la lipoperoxidación de sus membranas (Naha et al., 2005). El efecto oxidativo sobre estos procesos se analizan en el punto 4.3.

El metabolismo energético del espermatozoide que requiere mayor cantidad de ATP que otras células también es clave para conocer los procesos post-testiculares y el posible efecto del Pb en los mismos. Por una parte se requiere energía para la hiperactivación y el mantenimiento de su motilidad, y esta se obtiene por la ruta de glucólisis que se realiza en la zona del flagelo. El resto de ATP necesario para la fosforilación de proteínas que tiene lugar durante el proceso de capacitación se obtiene por la ruta de fosforilación oxidativa (respiración mitocondrial) que se lleva a cabo en la pieza intermedia (Urner y Sakkas, 2003; Miki, 2007). En este último aspecto hay que destacar la alteración producida por el Pb de algunos procesos de fosforilación celulares

como resultado de la interferencia, por competencia con el Ca^{2+}, en la actividad de las enzimas proteín kinasas (Sun et al., 1999).

4.2.1. Pb y estrés oxidativo. Influencia en la composición y permeabilidad de la membrana del espermatozoide

La situación de estrés oxidativo se produce cuando en el metabolismo celular se altera el balance entre la producción de ERO y la capacidad antioxidante de la célula como consecuencia de una excesiva producción de estas especies como consecuencia de factores ambientales como es estilo de vida, o exposición a metales entre otras causas provocando que las defensas antioxidantes endógenas y exógenas no sean capaces de eliminar su acción dando lugar a daños celulares y titulares (Aseervatham et al., 2013). Como radicales libres se conoce a moléculas formadas durante el metabolismo oxidativo y que incluyen a átomos o moléculas en forma iónica que presentan uno o más electrones desapareados en su estructura, lo que les otorga una configuración espacial de elevada inestabilidad que les hace altamente reactivos con una amplia variedad de compuestos que en principio estables pero que al ser atacados quedan a su vez trasformados en compuestos reactivos provocando con ello una reacción de oxidación en cadena. Esta situación es muchas veces provocada por la exposición a metales pesados que provocan daños en las bases nucleotídicas del ADN, desencadenan la peroxidación de los lípidos de las membranas o llevan a alterar la homeostasis del Ca^{2+} y de los grupos sulfidrilos de las proteínas (Valko et al., 2005). Como ya se ha comentado uno de los posibles mecanismos de toxicidad del Pb que está detrás de la alteración de la función reproductora es la aparición del estrés oxidativo determinado

por una alteración del balance redox, como se observa en tejidos de ciervos de las áreas mineras (Reglero et al., 2009a).

La alteración del balance REDOX general como la consecuencia de la presencia del Pb y que llevaría a afectar a la función reproductora puede ocurrir a diferentes niveles (Figura 5):

- La inhibición de la enzima deshidratasa del ácido δ-aminolevulínico (δ-ALAD) lleva a la acumulación de su sustrato, el ácido δ-aminolevulínico (δ-ALA), que a su vez oxida a otras moléculas y produce radicales libres como el anión superóxido ($O_2^{\cdot-}$) radical hidroxilo (OH^-) y peróxido de hidrógeno (H_2O_2) (Ahamed et al., 2006).
- Actuación directa del Pb sobre el ión ferroso (Fe^{2+}) que inicia la peroxidación de las membranas celulares (Adonaylo y Oteiza, 1999).

Frente a la presencia de las EROs, el organismo pone en marcha un sistema antioxidante que incluye fundamentalmente compuestos producidos de forma endógena y que constituyen el primer nivel de defensa antioxidante tales como un grupo de enzimas que reparan daños oxidativos entre las que están la glutatión peroxidasa (GPX), la superóxido dismutasa (SOD), la catalasa (CAT) y la glutatión reductasa (GR). Además actúan un grupo de metaloproteínas no enzimáticas tales como las metalotineínas o la ferritina que se pueden considerar como antioxidantes al actuar eliminando o incluyendo en su molécula a precursores de radicales libres (metales de transición como el Fe o el Cu) o desactivando rutas metabólicas que los forman como es la reacción de Fenton (Surai, 2003). Por otra parte, a nivel también endógeno actúan antioxidantes de bajo peso molecular como el glutatión reducido y oxidado (GSH-GSSG), la vitamina C y el y los nucleótidos de nicotinamida y adenina (NADH y NADPH) que actúan como coenzimas en procesos redox, entre otros. En el caso de que

este mecanismo antioxidante sea insuficiente, la célula pone en funcionamiento un segundo nivel de defensa contra el daño oxidativo que es de origen exógeno al estar constituido por antioxidantes aportados por la dieta entre los que están los carotenos y las vitaminas A, E y C en cuya actividad interfiere el Pb como ya se ha comentado anteriormente. (Pérez-Rodríguez, 2009).

El GSH es un tripéptido constituido por glicina, cisteína y ácido glutámico que utiliza el grupo tiol (-SH) de la cisteína como agente reductor. Representa el tiol no proteico más abundante en las células animales y se encuentra en gran parte acumulado en el hígado. Actúa por ello como el principal regulador del estado redox de la célula, de modo que un pequeña modificación en la ratio entre su forma reducida (GSH) y oxidada (GSSG) refleja alteraciones celulares por estrés oxidativo (Lu, 2009).

La SOD es una metaloenzima con dos isoformas: Cu/Zn-SOD en el citoplasma o Mn-SOD en la mitocondria, responsable la reacción de dismutación en la que se trasforma el $O_2^{-\cdot}$ en oxígeno molecular y peróxido de hidrógeno que posteriormente será eliminado por la catalasa en los peroxisomas (Mruk et al., 2002).

La GPX es una enzima citoplasmática Se-dependiente que elimina el peróxido de hidrógeno trasformándolo en agua, utilizando para ello el GSH como sustrato (Cnuben et al., 2001). Por otra parte el selenio de forma independiente se puede considerar como un micronutriente antioxidante al tener un efecto positivo en la actividad del GSH y de la SOD.

Hay numerosos datos que apuntan al papel del Pb como disruptor del equilibrio oxidante-antioxidante de la célula. También se ha encontrado una relación positiva entre los niveles de la enzima antioxidante catalasa (CAT) muy activa en el plasma seminal y la motilidad individual y progresiva de los espermatozoides (Zelen et al., 2009), así como una correlación positiva entre la cantidad total de antioxidantes (TAS), SOD y

albumina en plasma seminal de herbívoros con buena motilidad (Tvrda et al., 2012). Por todo ello, hay estudios que muestran que mantener unos correctos perfiles de antioxidantes liposolubles en plasma y semen, representados por los carotenoides, la vitamina A y la E es importante para la fertilidad masculina (Benedetti et al., 2012). De la misma manera y como ya se comentado, en los ungulados de zonas mineras pertenecientes al área de estudio de esta tesis con exposición aguda y crónica al Pb se ha visto alterado el equilibrio antioxidante en diferentes tejidos, con una disminución de los niveles de glutatión hepático (GSH) (Reglero et al., 2009a), de los niveles de vitamina A y E en el hígado y testículo de estos animales (Rodriguez-Estival et al., 2011). La importante actividad como antioxidante endógeno del glutatión está afectada por la presencia de Pb (Gurer y Ercal, 2000), contrastada también en estudios *in vitro* (Pérez et al., 2013) puede ser debido en parte a la afinidad que presenta este metal por los grupos sulfhidrilos (-SH) (Kendall et al., 2001) que también forman parte de bastantes proteínas y enzimas que actúan en la célula como defensa antioxidante.

La capacidad fecundante del espermatozoide depende de que pueda lograr su estado de capacitación, que incluye la hipermotilidad del mismo y la liberación de las enzimas del acrosoma así como de completar la unión del pronúcleo masculino con el del ovocito tras penetrar en la zona pelúcida que rodea a éste. La reorganización en la composición y el mantenimiento de la estructura de la membrana del espermatozoide que tienen lugar durante estos procesos tienen un papel crucial en la fertilización (Tapia et al., 2012; Flesch y Gazella, 2000). El Pb puede afectar a la permeabilidad y selectividad la membrana en base a varios factores:

- A la modificación del perfil de ácidos grasos, muchas veces provocado por estrés oxidativo (Reglero et al., 2009a; Slaba et al., 2013).
- A una alteración del potencial de membrana (Kurtyka et al., 2011).

- A la unión de estos iones a las proteínas transportadoras de canal y competencia con el Ca^{2+} en las bombas de iones (Evans et al., 2003).

La influencia del Pb relativa a la alteración de potencial de membrana y la unión a proteínas trasportadoras se citan en los puntos 4.4 y 4.5.

El daño oxidativo cuando hay un desequilibrio en el equilibrio redox por efecto del Pb afecta fundamentalmente a las membranas (Fig. 5), al ser estás especialmente sensibles por la presencia de dobles enlaces en sus ácidos grasos de sus fosfolípidos, y queda patente por el aumento de malondialdehido (MDA) como producto de la lipoperoxidación de las mismas (Singh et al., 2013). El aumento de este marcador de peroxidación también se ha encontrado en espermatozoides de rumiantes con niveles en plasma seminal de 230 ppb de Pb^{2+} que contaban con una defensa antioxidante alterada por la disminución la catalasa (CAT) y el glutatión reducido (GSH) y que afectó negativamente a la calidad espermática (Tvrda et al., 2012). Por otra parte, la composición lipídica de la membrana del espermatozoide es un factor importante para la viabilidad, maduración y funcionalidad de los mismos. En el caso concreto de bovinos el ácido graso mayoritario de los fosfolípidos de la membrana el ácido docosahexaenoico (DHA, 22:6n-3), seguido del ácido araquidónico (20:4n-6), si bien durante la maduración de espermatozoides en el epidídimo se observa un aumento en la cantidad de ácido araquidónico, docosapentaenoico (DPA, 22:5n-6) y DHA(Neill y Master, 1972; Rejraji et al., 2006). También se produce una liberación de colesterol de las membranas durante el proceso de capacitación (Tapia et al., 2012). A este respecto que hay autores que encuentran una relación entre bajas ratios colesterol/fosfolípidos y aceleración de la capacitación y así, en el caso de moruecos la ratio es de 0,37 y su tiempo de capacitación es 1,5 h mientras que en humanos es de 0,99 con un tiempo de capacitación de 7 horas (Davis, 1981).

El mantenimiento de una proporción elevada de PUFAs es importante para mantener la capacidad fecundante (Aksoy et al., 2006), del mismo modo que el aumento de ácidos grasos saturados aparece ligado a los casos de infertilidad (Tavilani et al., 2006). Del mismo modo, se ha mostrado una correlación positiva entre la cantidad de ácidos poliinsaturados n-3 de cadena larga, en concreto del DHA, y la motilidad, concentración espermática y ausencia de morfoanomalías.

Como consecuencia de la particular composición de la membrana del espermatozoide, rica en ácido grasos insaturados, ésta es más lábil al ataque de ERO, lo que puede provocar lipoperoxidación de la misma y una alteración de su composición y estructura.

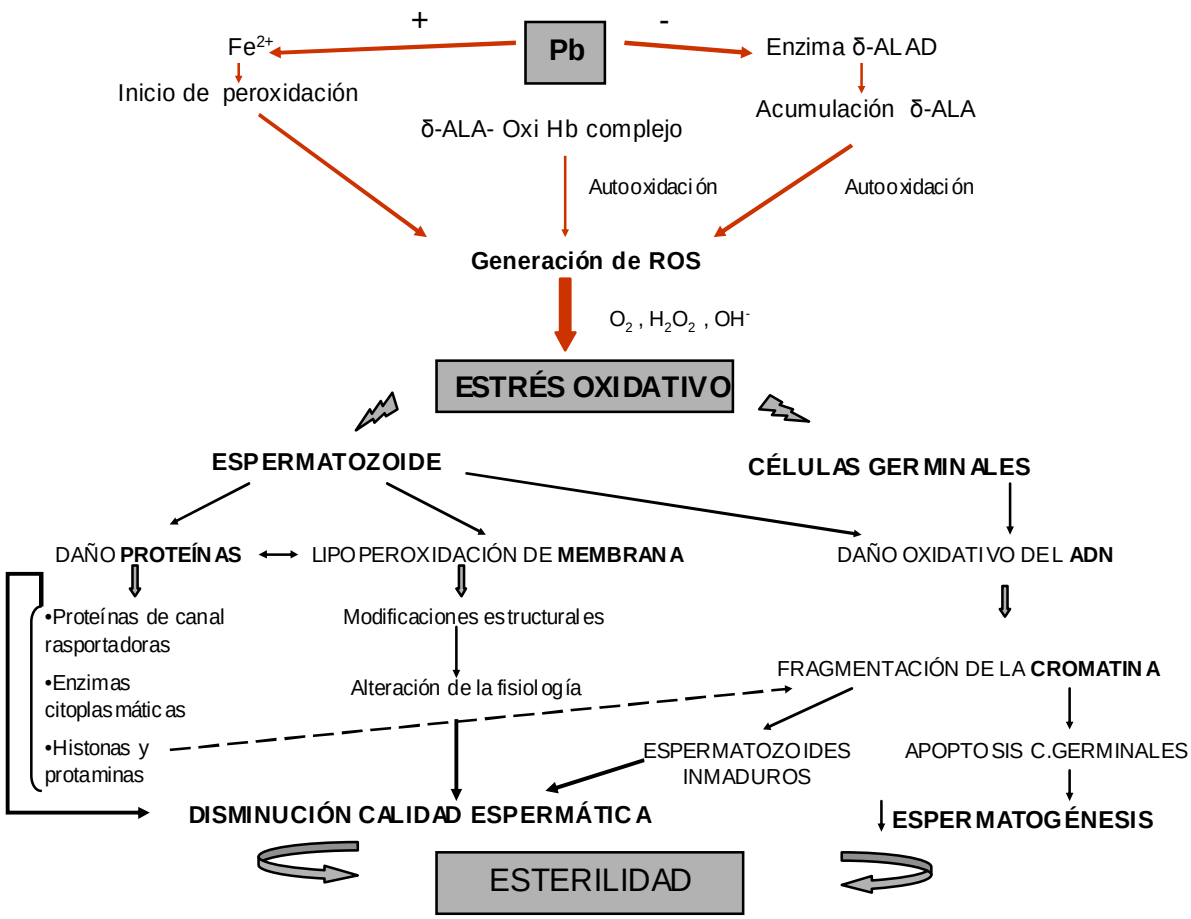

Figura 5: *Causas de estrés oxidativo por Pb y efectos en el espermatozoide*

4.2.2. Efecto del Pb sobre la motilidad

Se sabe que la fertilidad masculina está correlacionada con la tasa de penetración del espermatozoide en el mucus cervical, y que ésta a su vez depende de factores como la trayectoria inicialmente lineal del espermatozoide, de la velocidad de desplazamiento y de la amplitud del desplazamiento lateral de la cabeza, si bien por la multitud de factores que intervienen en este proceso, esta tasa de penetración no se puede tomar como parámetro definitivo para medir la probabilidad de fertilización (Beltsos et al., 1996). De cualquier modo, uno de los primeros factores necesarios para lograr la fertilización es que la célula adquiriera una motilidad hiperactiva (Suarez, 2008), caracterizada por una elevada velocidad y ondas flagelares asimétricas que permitan que el espermatozoide se separe del epitelio del oviducto y atraviese finalmente la zona pelúcida que rodea al ovocito. Por ello, esta motilidad hiperactiva resulta más importante para predecir la capacidad potencial de fertilización del espermatozoide (Cancel et al., 2000). Hay que tener en cuenta que durante el proceso de maduración y almacenamiento del espermatozoide en los epidídimos su membrana permanece estructural y funcionalmente estable, pero cuando el espermatozoide inicia proceso de capacitación en las vías reproductoras masculinas y que finaliza en el aparato reproductor femenino, tienen que tener lugar importantes procesos de reorganización en la estructura y fisiología de la membrana que le permitan en primer lugar adquirir esta hipermotilidad y finalmente la exocitosis acrosomal para conseguir su fusión con el ovocito. Uno de los principales factores que regulan los cambios en el espermatozoide para conseguir esta hipermotilidad y la posterior capacitación es la elevación de la concentración intracelular de Ca^{2+} (Carlson et al., 2007, Breitbart y Naor, 1999), ya que de su incremento en el citosol depende tanto la fosforilación de proteínas que

condiciona el inicio del proceso de glucólisis en el axonema (Arcelay et al., 2008), como los cambios en el potencial de membrana que se realizan a la vez y que llevan a la hiperpolarización de la misma (Visconti et al., 2011). Aunque son muy variadas las proteínas que sirven de sustrato para la fosforilación de la tirosina, entre las que están la tubulina y la acrosina, se han identificado dos aldolasas específicas de testículo que sufren este proceso de fosforilación (Arcelay et al., 2008). Por otra parte, estos procesos de fosforilación de proteínas están mediados por nucleótidos cíclicos como el adenosín monofosfato (AMPc), que son Ca^{2+}-dependientes (Visconti et al., 2011; Demarco et al., 2003). Por ello, el efecto del Pb sobre la motilidad puede deberse a la competencia de este metal con el Ca^{2+} en los procesos fisiológicos de la membrana que intervienen en el trasporte de iones (Evans et al., 2003, Chen et al., 2009), que llevaría a modificar la homeostasis del Ca^{2+} en el citosol y con ello a todos los procesos dependientes del mismo, como puede ser la adquisición y el mantenimiento de la hipermotilidad. De esta forma, hay estudios que muestran los efectos intra y extracelulares del Pb por interacción con proteínas de membrana que son receptores del Ca^{2+}, como es la calmodulina, y con canales de potasio que son dependientes del Ca^{2+} (Sun et al., 1999). Por otra parte, la alteración en el trasporte de iones puede deberse a algunos de los daños oxidativo producido por el Pb sobre las membranas, como es la oxidación de los grupos tiol a disulfuros (-SH a -SS) en las proteínas de las mismas (Seligman et al., 2004). Por otra parte, para mantener tanto la motilidad como la viabilidad del espermatozoide es necesario un nivel adecuado de prostaglandinas en el plasma seminal que son formadas por la próstata (Yeste et al., 2008). Estas prostaglandinas son un producto del metabolismo oxidativo del ácido araquidónico por vía de la enzima ciclooxigenasa (COx) (Breitbart y Spungin, 1997). El Pb puede interferir en la producción de las mismas por mecanismos de estrés oxidativo como consecuencia de

una disminución del ácido araquidónico en las membranas. Este efecto del Pb sobre la secreción de prostaglandinas también se ha observado en células somáticas en las que la concentración de 1 μM Pb^{2+} actúa sobre la secreción de prostaglandinas modificando la regulación de la trascripción de los genes estructurales de la COx (Chang et al., 2011).

4.2.3. Efecto del Pb sobre la reacción del acrosoma

La unión final de los dos gametos durante el proceso de fecundación requiere unos procesos secuenciales destinados a la penetración del espermatozoide en las glicoproteínas de la zona pelúcida (ZP) que rodea al ovocito y de la preparación de su superficie para la fusión con el mismo. Para que estos mecanismos que están muy interrelacionados se lleven a cabo se desencadena en el espermatozoide un proceso de exocitosis desde la parte anterior de la cabeza (acrosoma) conocido como reacción del acrosoma, que permite la liberación de un grupo de enzimas cuya finalidad última será la fusión de los dos gametos. La fecundación no se podría llevar a cabo en el caso de que el espermatozoide perdiera la integridad de su acrosoma antes del contacto con el óvulo. Las enzimas liberadas durante la reacción del acrosoma son enzimas proteolíticas que actúan primeramente sobre los proteoglicanos de la ZP y constituyen un complejo sistema en el que participan la acrosina y espermosina (Kim et al., 2008). Posteriormente actúa la hialuronidasa encargada de despolimerizar el ácido hialurónico del espacio que existe entre las células corticales de la periferia del ovocito. Tras el ablandamiento de la ZP por las enzimas liberadas en la zona de interacción de los dos gametos, el contacto de la cabeza del espermatozoide origina más tarde un endurecimiento del resto de la ZP para evitar la poliespermia que unida a la pérdida

local del grosor de esta zona y a la hiperactivación del espermatozoide permitirá la unión final del pronúcleo masculino y femenino (Guyton. 1995).

La reacción del acrosoma es el resultado último de una serie de cambios en la membrana y citosol del espermatozoide ligados siempre a un aumento en los niveles de Ca^{2+} y de pH intracelular y que muchas veces está condicionado por el contacto con secreciones uterinas (Lishko et al., 2010). Para ello hay una modificación de los canales de la membrana trasportadores de iones que se inicia con una liberación de colesterol desde la misma y que provoca un aumento de su fluidez, favoreciendo a su vez la modificación del trasporte de iones en la membrana y la hiperpolarización de la misma (Tapia et al., 2012). Unido a lo anterior, a nivel citosólico continúan los procesos de fosforilación de proteínas en la que participan enzimas como la proteinkinasa C (PKC) y la proteinkinasa A (PKA). Éstas a su vez estimulan los canales de Ca^{2+} de la membrana interna acrosomal y provocan un nuevo aumento del Ca^{2+} intracelular que llevará al final a la liberación de las enzimas del acrosoma (Demarco et al., 2003; Visconti et al., 2011; De la Vega-Beltrán et al., 2012). Por otra parte, estas enzimas activan a las enzimas fosfolipasas A (PLA) y fosfolipasa C (PLC) encargadas de movilizar a los fosfolípidos, contribuyendo a su vez al incremento de fluidez de la membrana (Fig.6).

Por otra parte, es bien conocido que las bombas de iones actúan de diana para los metales pesados actuando éstos como potentes inhibidores de las bombas de Ca^{2+} e incluso a bajas concentraciones alteran la homeostasis del Ca^{2+} a nivel intracelular y actúan sobre las funciones celulares mediadas por el mismo (Chen et al., 2009)**.** En el caso del Pb, éste actúa con una inhibición competitiva altamente selectiva sobre los canales de Ca^{2+} llegando a desplazar a este ion en el centro activo a concentraciones micromolares de Pb (Vijverberg et al., 1994), lo que viene a reforzar la alteración de los

mecanismos de transporte de Ca^{2+} en la membrana del espermatozoide ya comentados. Por otra parte, el Pb a nivel de membrana, actúa mediante la interacción con los canales de K^{+}-Ca^{2+} dependientes, como se ha demostrado en otros estudios en los que individuos con infertilidad a consecuencia de la exposición al Pb muestran una disminución en la capacidad de conseguir la reacción del acrosoma **(**Benoff et al., 2000).

Otro de los efectos del Pb en este proceso puede producirse por interacción, bien de forma directa o a través de mecanismos de estrés oxidativo (Fig.6), con enzimas citosólicas como la PKC (Long et al., 1994), enzima que estimula a su vez la actividad de la PLA (Breitbart et al., 1997), encargada de liberar hacia el citosol el ácido araquidónico de las membranas. Como se ha comentado, este compuesto es trasformado por la COx en prostaglandinas y por la lipooxigenasa (LPOx) en ácido 15-hydroxyl-5,8,11,13 eicosatetraenoico (HETE), y ambas moléculas participan activamente en la exocitosis acrosomal (Breitbart y Spungin, 1997).

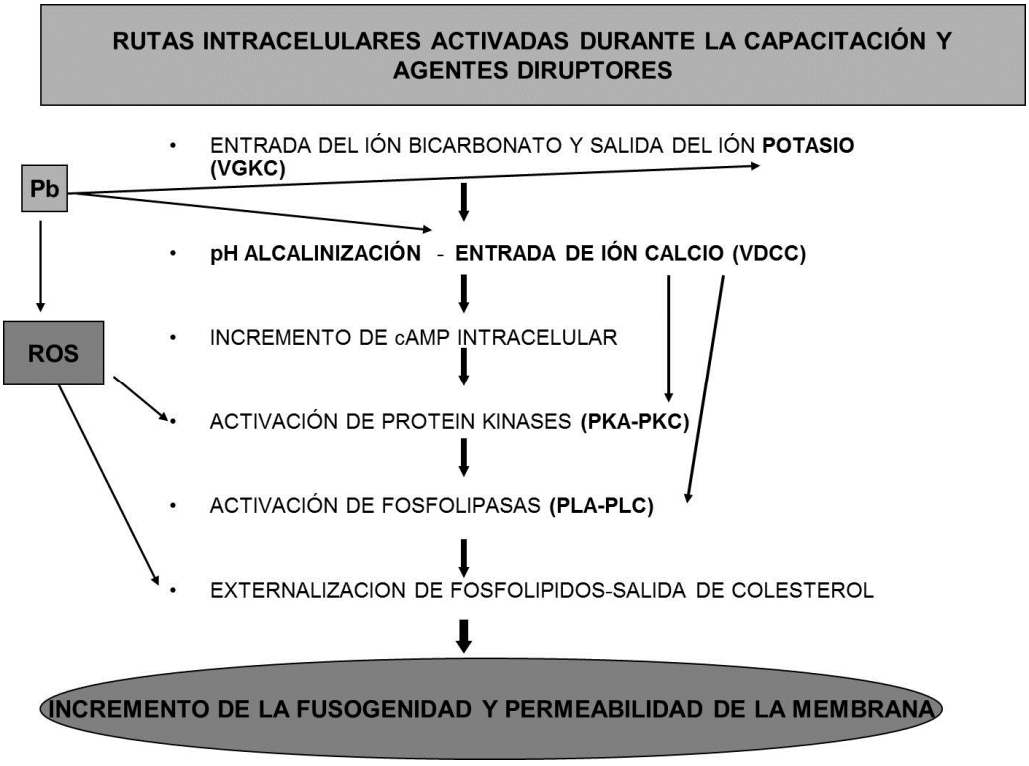

Figura 6: *Efecto adverso del Pb en la capacitación y reacción del acrosoma*

4.3. Genotoxicidad del Pb. Efecto sobre la estabilidad de la cromatina.

La molécula de ADN puede sufrir fragmentación en su hélice y con ello una relajación en el enrollamiento de las dos cadenas nucleotídicas como consecuencia de cambios drásticos de pH, choques térmicos o exposición a agentes tóxicos responsables de daños oxidativos. Estos daños generan productos potencialmente mutagénicos como la 7,8-dihydro-8-oxoguanina (8-oxodG), una forma oxidada de la base desoxiguanosina del ADN (Taggart et al., 2014). La 0-oxodG es utilizada como parámetro bioquímico de daños oxidativos en el ADN ya que contribuye al proceso de fragmentación de la cromatina (Abd-Allah et al, 2009). Son muchos los estudios que han asociado la disminución en la fertilidad que presentan muchas especies con el daño oxidativo del ADN (Aitken y Sayer, 2003; Gillan et al., 2005), así como la influencia del mantenimiento de la integridad a nivel testicular del ADN para asegurar una correcta espermatogénesis (Fraczek y Kurpisz, 2005) (Fig. 5).

Hay suficientes datos que muestran el efecto carcinogénico del Pb por daños en el ADN en diferentes tipos celulares, tanto en animales como en vegetales. Uno de los posibles mecanismos que pueden llevar al efecto genotóxico de este metal puede deberse a la interacción a nivel molecular con otros metales divalentes, en este caso con el Zn que interviene en los proceso de empaquetamiento del ADN en el espermatozoide y que se comentará más adelante. El Zn forma parte también de algunas proteínas que actúan bien como factores de trascripción de genes o intervienen en el mecanismo de reparación de la hebra de ADN, de modo que una sustitución del Zn por Pb estaría en la base de la alteración molecular de la expresión génica, tal y como muestran estudios donde hay una correlación entre exposición al Pb y daños en el ADN en diferentes células (Danadevi et al., 2003; Davidson et al., 2007). Este efecto directo del Pb sobre el

ADN también se ha estudiado *in vitro* en ratones tratados por vía oral con este metal y que llegaron a tener niveles sanguíneos del mismo entre 10-50 µg/dl, en los que el Pb no absorbido en el tracto digestivo alteró las células de la mucosa intestinal al actuar sobre la expresión génica (Breton et al., 2013).

Por otra parte, la genotoxicidad del Pb puede estar muchas veces mediada por mecanismos de estrés oxidativo derivados de la exposición al mismo, ya que hay estudios en los que se relaciona la presencia de ERO con daños en el material genético (Xu et al., 2003; Kiziler et al., 2007). Estos daños también se encuentran en ocasiones asociados a alteraciones estructurales en las membranas de diferentes tipos celulares como se muestra en hombres con espermatozoides de baja motilidad y que presentan parámetros de estrés oxidativo junto a una mayor proporción de cromatina fragmentada (Barroso et al., 2000). Igualmente, en estudios de infertilidad masculina se han encontrado ERO en plasma seminal junto con mayor nivel de fragmentación del ADN, tanto en espermatozoide como en células somáticas del eyaculado, y esto unido en ocasiones a células apoptóticas micro y binucleadas o con aberraciones cromosómicas (Marchetti y Marchetti. 2005).

Para conocer la incidencia del grado de fragmentación de la cromatina del espermatozoide en la infertilidad masculina, en los últimos años se han puesto a punto nuevas técnicas para analizar el estado estructural de la cromatina y determinar el índice de fragmentación del ADN. Otro factor tener en cuenta es que el grado de empaquetamiento que ha de presentar el ADN en el nucleosoma del espermatozoide es muy superior al que presentan las células somáticas con el objetivo de preservar la información genética del ataque de tóxicos hasta que se produzca la fecundación (Bungum et al., 2012; Giwercman et al., 2010). Así, una disminución prematura de la estabilidad de la cromatina incrementa el riesgo de daño al ADN por agentes

genotóxicos y podría poner en riesgo la capacidad fecundante de la célula (Björndahl y Kvist, 2014). Por otra parte, durante la espermatogénesis se ha de mantener un equilibrio entre el número de células germinales que entran en meiosis y el de células de Sertoli. Para ello, durante la espermatogénesis se produce la apoptosis natural de algunas células germinales , si bien un incremento de la tasa de apoptosis natural deriva en una disminución de la fertilidad (Dunkel et al., 1997; Kierszeubaum et al., 2001). Es por ello que el ataque de tóxicos como el Pb podrían afectar a este equilibrio aumentando la tasa de fragmentación de la cromatina y con ello el número de células apoptóticas (Marchetti y Marchetti, 2005).

Los efectos del Pb sobre la cromatina del espermatozoide puede ser aún más marcado que en otras células ya que en él hay necesidad de un empaquetamiento eficaz del ADN que se refleja en sus particularidades estructurales. En el espermatozoide la cromatina tiene una doble función que es por una parte, proteger de los daños en el ADN durante el almacenamiento y trasporte hasta el ovocito, y por otra facilitar un buen desembalaje del ADN indemne tras la penetración del ovulo. Esto supone el embalaje del filamento del ADN alrededor de estas proteínas básicas, que además neutralizan la acidez de la molécula, como son proteínas histonas y protaminas. En este proceso, el zinc tiene una importante función. En relación a las histonas, la H1 que influye en el plegamiento actuando como conexión entre nucleosomas, tienen variantes en la línea germinal. En concreto la H1LSI participa en la condensación nuclear durante la elongación de las espermátidas, mientras que la H1T2 es esencial para mantener la compactación y polarización de la cromatina en las últimas etapas. Esta estructura se logra después de una serie de cambios estructurales en la unidad de empaquetamiento de la cromatina (nucleosoma) que tienen lugar durante la espermatogénesis y que hacen más sensible al ADN a daños oxidativos. En estos cambios se van sustituyendo las

proteínas histonas somáticas por proteínas de transición (TP1 TP2) y después por protaminas (prot 1 y prot 2) que son las proteínas mayoritarias en el ADN de los espermatozoides y últimas responsables del empaquetamiento máximo del mismo. De este modo, la alteración en la composición o deficiencia en las mismas está correlacionada con esterilidad masculina como muestra el hecho de que en hombres fértiles las cantidades de prot 1 y prot 2 son similares y se mantienen constantes, a diferencia de lo que ocurre en casos de esterilidad (Jodar y Oliva, 2014). Por otra parte, como las protaminas son muy ricas en los aminoácidos arginina y cisteína, usados para unirse a las protaminas vecinas mediante puentes disulfuro entre grupos sulfidrilos (Kanippayoor et al., 2013), esta proteína resulta ser especialmente sensible al ataque por Pb debido a la afinidad de este metal por estos grupos. Por este motivo, la presencia de Pb durante el proceso de espermatogénesis podría poner en riesgo el proceso de empaquetamiento de la cromatina, dejando al filamento de ADN más lábil frente al ataque oxidativo derivado de la presencia de este metal.

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Objetivos

Como se ha explicado en la introducción general, el plomo es un metal con constantes aportaciones antropogénicas al medio ambiente a lo largo de la historia al ser muy utilizado por diferentes civilizaciones, aportaciones que se han acrecentado desde la época preindustrial hasta nuestros días. Todo esto lleva parejo no solo el serio deterioro ambiental que han supuesto las actividades mineras de extracción de este metal así como los residuos generados de la misma, sino también efectos muy negativos sobre la salud humana que han sido observados en múltiples estudios. La toxicidad del plomo sobre el sistema reproductor es uno de los aspectos que más preocupan en la actualidad al verse relacionado con muchos problemas de esterilidad humana, aún a niveles de concentración de plomo en plasma sanguíneo y semen que se asocia a efectos subclínicos.

En muchas áreas del Valle de Alcudia y Sierra Madrona y como consecuencia de la existencia de entornos mineros abandonados y sin recuperar resultado de la intensa actividad minera mantenida hasta la segunda mitad del siglo pasado, se acumulan unos niveles en suelo de este metal que son superiores a la media europea. Esto lleva a una exposición continua al plomo y otros metales y metaloides, como son el cobre, selenio, zinc o arsénico, a la que están sometidos los ungulados que se alimentan en estas zonas, y en concreto el ciervo común. Esto que queda evidenciado en estudios anteriores en los que se muestran unas concentraciones de plomo en tejidos como hígado, hueso o bazo que son superiores a los de otros animales de zonas control sin suelos contaminados. Es por ello que para aproximar los posibles efectos que puede tener el

plomo y otros metales así como la interferencia entre los mismos sobre la fertilidad del ciervo común, el *objetivo general de toda esta tesis es el de evaluar los efectos tóxicos del plomo sobre la calidad espermática, tanto en estudios de campo, y por lo tanto in vivo, en esta especie silvestre (capítulos 1, 2 y 3), como estudiar sus efectos mediante ensayos experimentales in vitro con espermatozoides del morueco (capítulos 4, 5 y 6).* Estos ensayos *in vitro* han tenido como objetivo buscar un modelo de experimentación donde se puedan estudiar los efectos de este metal sobre el espermatozoide y que se puedan extrapolar a la fauna silvestre.

El efecto adverso del plomo sobre muchas funciones vitales se debe a una situación de estrés oxidativo derivado del desequilibrio entre prooxidantes-antioxidantes. El *primero de los objetivos específicos es determinar la relación entre la presencia de plomo y otros metales pesados en el testículo con la disminución de la calidad espermática y biomarcadores de estrés oxidativo y antioxidantes de los espermatozoides y de los testículos en los ciervos de las zonas mineras* (Capítulo 1).

El mantenimiento de la estructura y la función fisiológica de la membrana lipídica del espermatozoide es esencial para asegurar unos correctos parámetros de calidad espermática, como son motilidad, viabilidad de membrana e integridad del acrosoma. *El segundo objetivo específico es estudiar el efecto que el plomo ha podido tener en el perfil de ácidos grasos presente en testículos y espermatozoides de ciervos expuestos a contaminación minera,* que podrían estar relacionados con peroxidación de lípidos de la membrana y por lo tanto con el estrés oxidativo comentado en el objetivo anterior (Capítulo 2).

La exposición ambiental al plomo y otros metales pesados causa fragmentación del ADN de los espermatozoides que se relaciona con una pérdida de calidad seminal. *El tercer objetivo específico es determinar si los niveles de plomo que aparecen en el*

epidídimo, en el parénquima testicular y en espermatozoides de ciervos de áreas mineras han producido daños en la cromatina y si estos efectos están relacionados con alteración en la actividad de antioxidantes endógenos y la calidad de los espermatozoides (Capítulo 3).

El estudio *in vitro* de los efectos del plomo en los espermatozoides puede abrir nuevas vías para conocer los mecanismos bioquímicos involucrados en la toxicidad del plomo a nivel post-testicular. *El cuarto objetivo específico ha sido determinar el umbral de concentración de plomo y el tiempo de exposición a este metal que es capaz de afectar negativamente a la calidad espermática* con el fin de poder después usarlo en posteriores estudios *in vitro* (Capítulo 4).

Los efectos del plomo sobre la calidad espermática observados en estudios *in vivo* indican la posibilidad de efectos relacionados con la generación de estrés oxidativo y modificación del perfil de ácidos grasos de las membranas de los espermatozoides. *El quinto objetivo ha sido valorar el efecto del plomo in vitro en la calidad espermática, en biomarcadores de estrés oxidativo y antioxidantes y en la composición de ácidos grasos* (Capítulo 5).

Por último, otro de los mecanismos de toxicidad del Pb^{2+} en el espermatozoide es la interferencia con el Ca^{2+} en muchas funciones fisiológicas, como por ejemplo la reacción del acrosoma. *El sexto objetivo específico ha sido valorar in vitro el efecto que el plomo puede tener sobre los parámetros de calidad espermática, en especial la viabilidad de membrana y la integridad del acrosoma, mediante tres moduladores de proteínas de canal y citosólicas involucradas en la fisiología del* Ca^{2+} *y los mecanismos que desencadena* (Capítulo 6).

CAPÍTULO 1

Reduced sperm quality in relation to oxidative stress in red deer from a lead mining area

Escombreras de la mina de La Petaca (Valle de Alcudia) Foto: Vicente Luchena.

Reglero MM, Taggart MA, Castellanos P, Mateo R. 2009b. **Reduced sperm quality in relation to oxidative stress in red deer from a lead mining area.** *Environmental Pollution* 157: 2209-2215.

Reducción de la calidad espermática en ciervo común de área minera y su relación con estrés oxidativo

RESUMEN

Se han estudiado los efectos en la calidad del esperma de ciervo común de áreas mineras, de la elevada absorción de metales pesados, así como los mecanismos antioxidantes en espermatozoides y testículo de estos animales. Para ello se han analizado las concentraciones de Pb, Zn, Cu, Cd, As y Se de un total de 21 animales de áreas mineras y 20 de áreas control. Los testículos han sido pesados y medidos y se han valorado la motilidad, la integridad del acrosoma y la viabilidad y funcionalidad de la membrana en los espermatozoides epididimarios además de analizar los niveles de peroxidación lipídica (TBARS), el glutatión total y oxidado (tGSH) y la actividad de las enzimas glutatión peroxidasa (GPx) y superóxido dismutasa (SOD) en testículos y espermatozoides. Los ciervos de zonas mineras en relación a los animales de las zonas control, han mostrado menos concentración de Cu en el testículo junto a una masa y tamaño superior además de una reducción de la viabilidad e integridad del acrosoma del espermatozoide. Por último, estos efectos sobre la calidad espermática se asociaron con una disminución del Cu y un aumento de Se en los testículos y con una disminución en la actividad de SOD y GPX tanto en los testículos como en los espermatozoides.

***Palabras clave*:** esterilidad masculina, antioxidantes, metales pesados, fauna silvestre.

ABSTRACT

We studied the effects of elevated heavy metal uptake on the sperm quality and the antioxidant mechanisms of sperm and testis of red deer from a Pb mining area in Spain Testis, liver and bone of red deer from mining (n=21) and control (n=20) areas were obtained from hunters and analyzed for Pb, Zn, Cu, Cd, As and Se. Testes were weighed and measured. Motility, acrosome integrity and viability and functionality of membrane were evaluated in epididymal spermatozoa. Lipid peroxidation, total and oxidized glutathione, glutathione peroxidase and superoxide dismutase were studied in testis and spermatozoa. Deer from mined areas showed less Cu in testis, a higher testis mass and size and reduced spermatozoa membrane viability and acrosome integrity. Effects on sperm quality were associated to decreased Cu and increased Se in testis, and to decreases in the activity of SOD and GPX in testis and spermatozoa.

***Keywords**: m*ale reproduction, sterility, antioxidants, heavy metals, wildlife.

1. Introduction.

Heavy metal mining and smelting commonly contaminates air, water and soil compartments and this contamination is then transferred to biota through food chains. The accumulation of toxic heavy metals in some plants increases the risk of transfer to livestock and game animals (Chaney, 1989). Moreover, exposure of wildlife species to heavy metals can occur through the direct ingestion of soil associated with food items (Beyer and Fries, 2003) or as certain species instinctively lick soil to attain essential elements (Beyer et al., 2007).

In the province of Ciudad Real in southern Spain, the extraction of argentiferous galena started at the beginning of the 1st millennium B.C., and has continued intermittently ever since, with peaks of extraction and smelting activity occurring at the end of the 2nd century B.C., the end of the 15th and during the 16th century, and finally during the 19th-20th centuries (Hevia, 2003). This province became the major Pb producing area in Spain during the second half of the 19th century, but the last workings closed in 1988. Around 484 old mines and prospects are located in an area spanning approximately 2500 km^2, the majority of which targeted Pb–Zn veins. Other metals were also produced in the area though, i.e, Ag, Cu, Sb, Sn, W, As and Bi (Palero-Fernández and Martín-Izard, 2005). The land affected by these mines has never been remediated, and heavy metal contamination has been reported in plants and animals sampled in the area (Reglero et al., 2008, 2009).

Although the Pb concentrations detected in liver of large game from mined areas in the Sierra Madrona were below the levels associated with clinical poisoning, red deer (*Cervus elaphus*) did have 39% less total glutathione (GSH) in liver, (than animals from control sites) indicating a measurable effect on oxidative stress due to metal exposure (Reglero et al., 2009). Heavy metal exposure induces oxidative stress in several tissues

and in cells, including testis and spermatozoa (Stohs and Bagchi, 1996; Mateo et al., 2003; Doreswamy et al., 2004; Marchlewizc et al., 2004; Subramanian et al., 2006). Oxidative stress affects male fertility because it damages sperm membranes, decreasing both sperm motility and its ability to fuse with the oocyte (Tremellen, 2008). Spermatozoa are especially susceptible to oxidative stress due to the high level of polyunsaturated fatty acids (PUFA) in their membranes (Vernet et al., 2004). In ram (*Ovis aries*) spermatozoa, 45% of fatty acids are PUFAs (Castellanos et al., 2008). The generation of reactive oxygen species in sperm of Pb-exposed mice has been linked to an increase in acrosome reacted spermatozoa (Hsu et al., 1998), suggesting that oxidative stress induced by Pb is implicated in premature capacitation.

Despite knowledge regarding the adverse effects of Pb and other heavy metals on male reproductive function (Xu et al., 2003; Doreswamy et al., 2004; Marchlewizc et al., 2004; Subramanian et al., 2006), surprisingly few studies have been reported in wildlife (Blottner et al., 1999; Dauwe et al., 2004; Guillette et al., 2008). The key aim of our research was therefore to study the effect of elevated heavy metal uptake on the sperm quality and the antioxidant mechanisms of sperm and testis of red deer from mined areas in the Sierra Madrona, which are now largely devoted to, and occupied by, hunting estates.

2. Materials and Methods.

2.1. Study area.

The Sierra Madrona is in the northern part of the Eastern Sierra Morena in the Province of Ciudad Real, Southern Spain. The elevation here ranges between 500 and 1,300 m. The climate is Mediterranean sub-humid with marked seasons, and Mediterranean shrubland is dominant. During the sampling year (2005) Spain had the lowest annual rainfall since 1947, with only 279 mm in the Guadiana basin, where the study area is located (Spanish Meteorological Agency, http://www.aemet.es). The most important geological features in this area are a series of major Hercynian WNW–ESE trending anticlines and synclines. The lithostratigraphic sequence is composed of siliciclastic rocks with some interlayered volcanics and rare carbonate levels (Palero-Fernández and Martín-Izard, 2005). The Montes de Ciudad Real region (where the control sites were located) has altitudes between 500 and 1000 m, has similar geomorphological, climatic and biogeographic characteristics to Sierra Madrona, and is considered to be part of the same natural macroregion (García-Rayego, 1994). However, this area has not been widely mined historically and is not known to be rich in mineralised zones with high metal contents. The densities of red deer in managed hunting estates in the Sierra Madrona area (18.3-32.4 deer/100 ha) are within the range found in the Montes de Ciudad Real and Toledo area (11.3-90.1 deer/100 ha) (Vicente et al., 2007).

Eight hunting estates were selected, six of them in mined areas within the Sierra Madrona, and two in control areas in the Montes de Ciudad Real. All sampling sites were in the Province of Ciudad Real (Fig. 1).

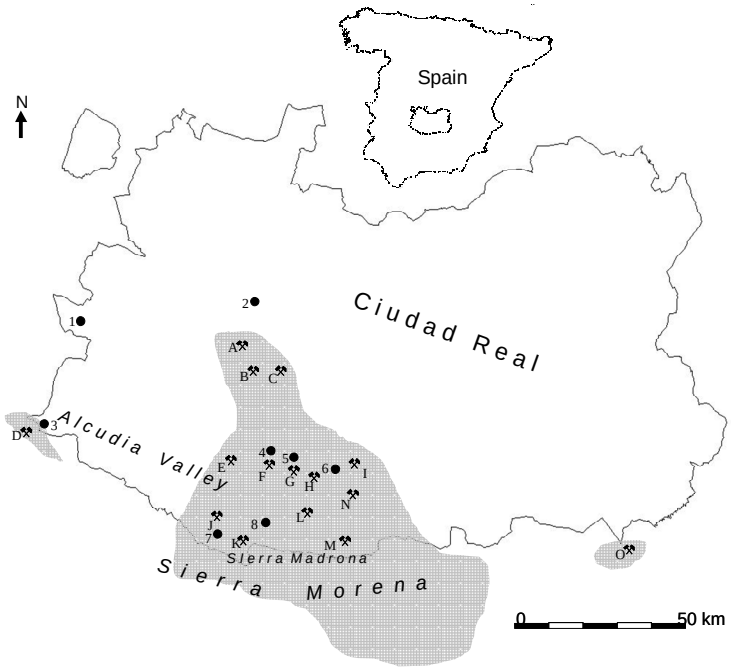


Fig.1. Study area in the province of Ciudad Real (S. Spain). The highest presence of Pb mines is found in the shaded area according to Palero-Fernández and Martín-Izard (2005). Some of the most relevant mines close to the sampling sites were Villagutiérrez (A) , La Victoria (B), San Quintín (C), Santa Catalina (D), La Romanilla (E), Cabezarrubias del Puerto (F), Hinojosas de Calatrava (G), Mestanza (H), Las Minillas (I), El Horcajo (J), Fuencaliente (K), Diógenes (L), Los Pontones (M), Los Galayos (N) and Los Engarbes (O). The sampled hunting estates were Lagunillas (1), Hornias Bajas (2), Los Baldíos (3), El Girote (4), Zamorillas (5), El Burcio (6), La Garganta (7) , Navalmartina (8).

2.2. Sampling.

Testes of 44 adult red deer were obtained from hunters during the autumn of 2005 in the mining (n=21) and control (n=20) areas. Sampling took place after "monterias" (large driven hunts), which usually last 3 h, at the end of which the deer are butchered and samples were taken during this process. Testes were transported to the laboratory within 1-2 hours of being removed from carcasses, and processed immediately upon arrival at the laboratory. The length, diameter and weight of the right and left testes of each deer were recorded. Spermatozoa were recovered from the distal portion of the epididymis by cutting the caudae epididymides with a surgical blade and

collecting oozing sperm mass as described by Soler et al. (2005), and placing it in 1 ml of phosphate saline buffer (pH 7.2; 8 g/l NaCl, 0.2 g/l KCl, 0.1 g/l $MgCl_2 \cdot 6H_20$, 0.1g/l $CaCl_2$, 1 g/l Na_2HPO_4, 0.15 g/l NaH_2PO_4, and 0.2 g/l KH_2PO_4) containing bovine serum albumin (at 5 g/l) and a final osmolality of 270-290 mOsmol/Kg. After spermatozoa collection, an inner portion of the parenchyma of the testis was frozen at -80ºC until analysis for oxidative stress biomarkers. Liver and bone sampling and processing for element analysis was previously described by Reglero et al. (2009).

2.3. Sperm quality measurements.

The individual motility, viability, and acrosome and plasma membrane integrities of spermatozoa were studied following the protocols described by Soler et al. (2005). Sperm concentration in the suspension was determined by microscopy in a Burker counting chamber. The % individual sperm motility was assessed in a small drop of sperm suspension held between a glass slide and a coverslip at 37 ºC under a phase-contrast microscope (magnification x400).

2.4. Analysis of oxidative stress biomarkers.

Lipid peroxidation estimated as thiobarbituric acid-reactive substances (TBARS) and reduced plus oxidized GSH (tGSH) were measured in homogenates of testis (0.5 g) or epididymal spermatozoa suspension (0.5 ml) following the methods described by Reglero et al. (2009). The activities of glutathione peroxidase (GPX; EC 1.11.1.9) and superoxide dismutase (SOD; EC 1.15.1.1) of the supernatant of testis and spermatozoa homogenates were measured by micromethods using the Ransel and Ransod kits

(Randox Laboratories). Enzyme activities were expressed relative to mg of protein in the supernatant of testis or spermatozoa homogenates (Reglero et al., 2009).

2.5. Elemental analysis.

The concentration of Pb, Cd, Cu, Zn, As and Se were determined in testes by atomic absorption spectroscopy as described by Reglero et al. (2009). Pb and Se were also determined in spermatozoa suspensions (0.4 ml) after digestion in quartz tubes with 2 ml of HNO_3 (65% Suprapur) for 12 h at room temperature. Then, 2 ml of H_2O_2 (30% v/v Suprapur) were added to each tube which was gradually heated in a thermoblock up to 150ºC for 4 h. Digested samples of spermatozoa were diluted to 5 ml with Milli-Q H_2O prior Pb and Se analyses (Reglero et al., 2009). The limits of detection in testis (LODs, in µg/g dry weight, back-calculated to in tissue concentrations) were for Pb: 0.058, Cd: 0.003; Zn: 1.5, Cu: 3.7, As: 0.06 and Se: 0.046. The LODs in spermatozoa were 1.45 and 11.3 pg/10^6 cells for Pb and Se, respectively. A reference sample of bovine liver (BCR 185R, Community Bureau of Reference) was analyzed (n=8) and the recovery was 94.4±5.8% for Pb, 95.8±0.7% for Zn, 100.4±0.6% for Cu, 99.0±4.0% for Cd, 114±11% for As and 74.3±2.2% for Se. A reference sample of pig kidney (BCR 186) was also analyzed in duplicate and the recovery for Se was 82.6%.

2.6. Statistical analysis.

Data below the detection limit were assigned values of half of the respective LOD for each element. The concentrations of the elements were log-transformed to attain or approach a normal distribution of the data. Differences in the concentration of

the studied elements in testis and spermatozoa suspensions between control and mining areas were analysed with generalized linear models (GLM), taking into account the hunting estate as a nested factor within the area. Oxidative stress biomarkers and the parameters of sperm quality were also compared between areas with nested GLM as described before. The relationships between the testis and spermatozoa concentrations for the different elements analysed, the oxidative stress biomarkers and the parameters of sperm quality were studied with linear correlations, and also, when correlations were more significant, with analyses of covariance including the area (mined or control) as a factor, and the element concentration as a covariant. Liver and bone levels of elements were also compared between areas for the studied males to assess if the same differences observed by Reglero et al. (2009) were also observed in the present subsample of adult males. Statistical significance was set at $p < 0.05$.

3. Results.

3.1. Differences between mining and control areas.

The concentration of Pb in testis of red deer males was not significantly higher in the mining area than in the control sites ($p=0.112$; Table 1), but detectable levels were only observed in the mining area. Moreover, males from the mining area had lower levels of Cu in testis than males from the control sites ($p<0.001$; Table 1). No significant differences were noted for Cd, Zn, As or Se in testis between mining and control areas, but differences were found among hunting estates (Table 1).

Table 1. Concentrations of elements in testis and epididymal spermatozoa of red deer

		Control area (n=20)		Mining area (n=21)		Nested GLM p values	
		Geometric mean (95% CI)	Range	Geometric mean (95% CI)	Range	Area	Estate[a]
Testis (µg/g dry weight)	Pb	ND	ND	0.037 (0.030-0.045)	ND-0.110	*ns*	*ns*
	Cd	0.007 (0.004-0.011)	ND-0.038	0.006 (0.003-0.010)	ND-0.033	*ns*	0.001
	Cu	8.32 (7.69-9.00)	6.09-10.45	6.21 (5.52-6.98)	3.79-8.98	<0.001	<0.001
	Zn	94.5 (91.9-97.2)	83.3-107.0	94.1 (91.5-96.8)	77.6-101.9	*ns*	<0.001
	As	0.065 (0.043-0.100)	ND-1.474	0.047 (0.045-0.050)	ND-0.070	*ns*	*ns*
	Se	1.09 (1.05-1.14)	0.91-1.35	1.09 (1.00-1.18)	0.07-1.42	*ns*	<0.001
Spermatozoa (pg/10^6 cell)	Pb	2.19 (1.39-3.44)	ND-50.42	2.97 (1.72-5.15)	ND-39.86	*ns*	*ns*
	Se	209.9 (169.7-259.5)	82.2-384.3	184.6 (149.8-227.4)	66.9-315.9	*ns*	*ns*

[a]Hunting estate was nested within area ; *ns* = not significantly different by area using a nested generalized linear model (GLM); ND: not detected

Lead and Se in the epididymal spermatozoa were not significantly higher in the deer from the mining area (Table 1). Liver Pb and Cu levels were significantly higher in males from the mining area than they were in control areas (mean (95%CI) in µg/g d.w.; Pb: 0.60 (0.32-1.12) vs. 0.71 (0.03-0.16), p=0.001; Cu: 53 (38-74) vs. 29 (20-42), p=0.025). Other elements concentrations in liver and bone did not differ between areas.

The length, diameter and mass of testes of red deer from the mining area were 6%, 9% and 24% higher than in deer from the control areas (Table 2). However, the percentages of epididymal spermatozoa with acrosome and membrane integrity were 11% and 15% lower in the mining area (Table 2). Most of the studied parameters, especially acrosome integrity and membrane viability, also differed among estates (Table 2).

Table 2. Characteristics of testis and epididymal spermatozoa of red deer

Parameter	Control area (n=20)	Mining area (n=24)	Nested GLM p values	
	Mean ± SE	Mean ± SE	Area	Estate[a]
Testis length (mm)	60.0 ± 1.5	63.7 ± 1.8	0.007	0.004
Testis diameter (mm)	33.9 ± 0.6	37.0 ± 1.0	<0.001	*ns*
Testis mass (g)	42.3 ± 2.4	52.5 ± 3.2	0.001	0.010
Concentration (10^6 spz/ml)	986 ± 154	1070 ± 140	*ns*	*ns*
Motility (%)	49 ± 7	54 ± 6	*ns*	0.001
Acrosome integrity (%)	90 ± 2	79 ± 3	0.018	<0.001
Membrane viability (%)	87 ± 2	72 ± 4	0.024	<0.001
Membrane functionality (%)	82 ± 2	74 ± 5	*ns*	0.044

[a]Hunting estate was nested within area; *ns* = not significantly different by area using a nested generalized linear model (GLM).

The level of tGSH was 15% higher in testis of red deer from the mining area than in the control area, and a significant difference also existed among hunting estates (Table 3). The activities of GPX and SOD were 16% and 14% lower in testis of red deer

from the mining area than in the control area (Table 3). The activity of SOD in epididymal spermatozoa was also 42% lower in deer from the mining area (Table 3). GPX activity in spermatozoa showed the same trend observed in testis with a lower value in the mining area, although the difference was not significant (p=0.079, Table 3).

3.2. Relationships between element concentrations, sperm quality and oxidative stress biomarkers

Concentrations in testis of Se were correlated positively with Zn (r=0.56, p<0.001). Cadmium was positively correlated with Pb (r=0.371, p=0.017) but negatively with Zn (r=-0.387, p=0.012). Other less significant correlations were observed in testis between As and Cu (r=0.33, p=0.035) and Zn and Pb (r=-0.317, p=0.044).

The concentration of Pb in spermatozoa was negatively correlated with Cu in testis (r=-0.334, p=0.035). No other significant relationships were noted between metals in testis and spermatozoa, likewise, no correlations were seen between levels of metals in spermatozoa.

The activity of SOD in testis was positively correlated with Cu (r=0.472, p=0.002); and, spermatozoa membrane viability was positively correlated with SOD activity (r=0.490, p=0.020) and Cu levels (r=0.802, p<0.001) in testis (Fig. 2). A significant correlation between testicular Cu level and acrosome integrity was also observed (r=0.664, p<0.001).

The activity of GPX in testis was positively correlated with the membrane viability of spermatozoa (r=0.582, p=0.004), but interestingly, Se levels in testis were negatively correlated with GPX activity (r=-0.391, p=0.014) in testis and membrane

viability of spermatozoa (r=-0.608, p=0.002; Fig. 3). Testicular Se was also negatively correlated with acrosome integrity (r=-0.488, p=0.001) and spermatozoa membrane functionality (r=-0.372, p=0.023). GPX activity and Cu levels were correlated in testis (r=0.421, p=0.008).

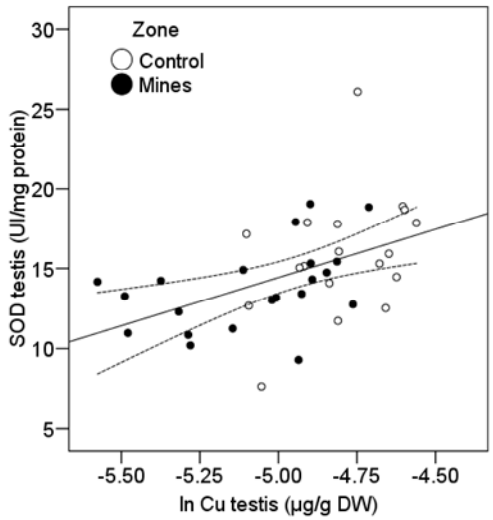
The mass of the testis was negatively correlated with testicular Cu (r=-0.426, p=0.005) and Se (r=0.379, p=0.015), and similar correlations were found between the length of testis and these elements. In contrast, testicular Zn was positively correlated with length (r=0.470, p=0.002) and mass of testis (r=0.335, p=0.032).

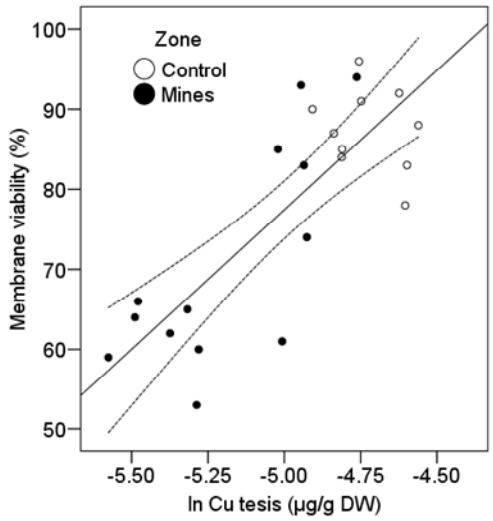
TBARS in spermatozoa were negatively correlated with Zn (r=-0.319, p=0.042) and Se (r=-0.453, p=0.003) in testis. GSH in testis was associated with spermatozoa motility (r=0.393, p=0.012) and acrosome integrity (r=0.394, p=0.012). Arsenic in testis was correlated with testicular SOD activity (r=0.496, p=0.001) and GSH in spermatozoa (r=0.353, p=0.023). Lead in spermatozoa was negatively correlated with spermatozoa viability (r=-0.421, p=0.045).

Table 3. Oxidative stress biomarkers in testis and epididymal spermatozoa of red deer.

		Control area (n=20)	Mining area (n=21)	Nested GLM p values	
		Mean ± SE	Mean ± SE	Area	Estate[a]
Testis	TBARS (nmol/g)	14.8 ± 0.7	15.0 ± 0.7	*ns*	*ns*
	tGSH (µmol/g)	1.81 ± 0.13	2.09 ± 0.13	0.006	<0.001
	GPX (mU/mg protein)	197 ± 11	165 ± 8	0.033	*ns*
	SOD (UI/mg protein)	16.1 ± 0.9	13.8 ± 0.6	0.016	0.026
Spermatozoa	TBARS (pmol/10^6 spz)	14.4 ± 3.2	11.0 ± 3.1	*ns*	0.004
	tGSH (nmol/10^6 spz)	0.041 ± 0.012	0.024 ± 0.004	*ns*	*ns*
	GPX (mU/10^6 spz)	7.44 ± 2.55	2.79 ± 0.82	*ns*	*ns*
	SOD (UI/10^6 spz)	0.130 ± 0.025	0.075 ± 0.009	0.031	*ns*

[a]Hunting estate was nested within area;*ns* = not significantly different by area using a nested generalized linear model (GLM).





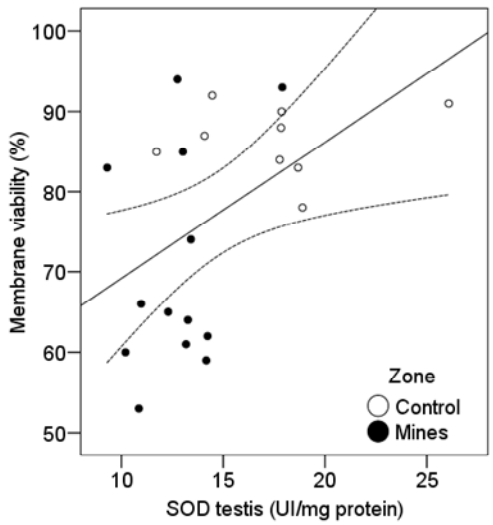
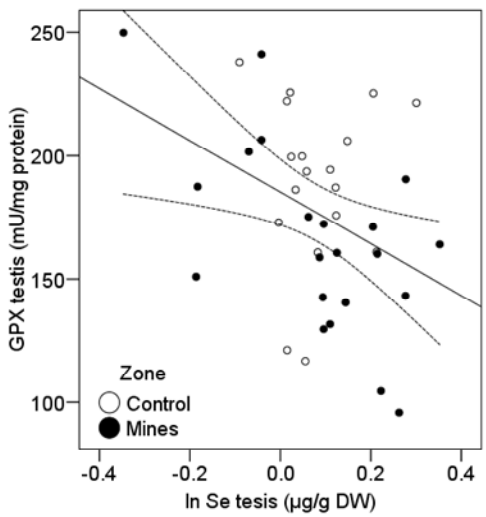
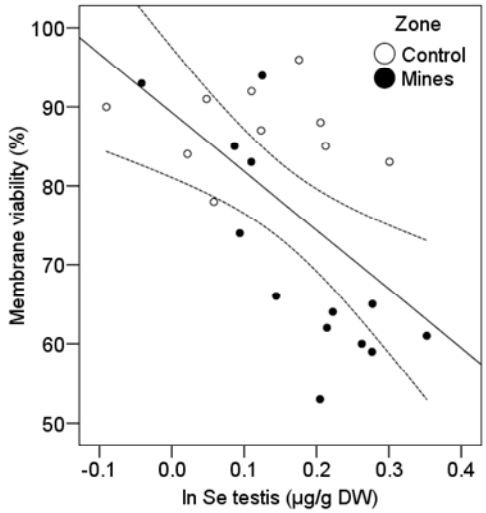


Fig.2. Relationship between Cu levels in testis, spermatozoa membrane viability and SOD activity in testis. (Top) In the ANCOVA analysis, SOD activity in testis was positively affected by Cu ($p=0.018$) with no effect of area (i.e, mined or control; $p=0.676$). (Middle) Spermatozoa membrane viability was positively affected by Cu ($p=0.001$), with no effect of area ($p=0.767$). (Bottom) The spermatozoa membrane viability was marginally affected by area ($p=0.055$), but not by the testicular SOD activity ($p=0.411$), although the correlation between spermatozoa membrane viability and SOD activity in testis was significant ($r=0.490$, $p=0.020$).





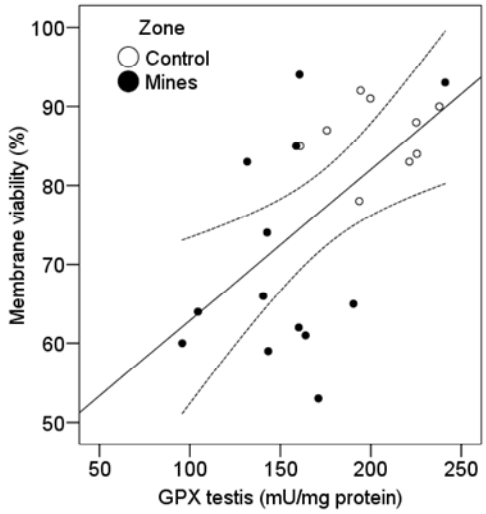


Fig.3. Relationships between Se levels in testis, spermatozoa membrane viability and GPX activity in testis. (Top) In the ANCOVA analysis, GPX activity was negatively affected by Se (p=0.01), with area (i.e., mined or control) having an effect (p=0.037). (Middle) Spermatozoa membrane viability was negatively affected by Se (p=0.005), and an effect due to area was also noted (p=0.004). (Bottom) The spermatozoa membrane viability was not significantly affected by area (p=0.099) or by the testicular SOD activity (p=0.131), although the correlation between spermatozoa membrane viability and GPX activity in testis was significant r=0.582, p=0.004).

4. Discussion.

Red deer from the Sierra Madrona mining area showed higher Pb levels in liver and bone than in control sites (Reglero et al., 2008, 2009), and this difference was also reflected in a trend to increase in Pb in the testis. Moreover, several parameters and biomarkers pertinent to male reproduction such as testis mass and size, membrane viability and acrosome integrity of spermatozoa and antioxidant enzyme activities in testis and spermatozoa differed between the mining and the control areas. These differences were not, however, directly associated with Pb levels in testis and sperm, but instead showed significant relationships with Cu and Se in testis and with antioxidant enzymes, such as SOD and GPX, which are associated with, and have a requirement for, these elements. Lead levels >5 µg/g d.w. in liver and >200 ng/ml in blood can be taken as chemical indicators of toxic exposure to Pb in mammals (Ma, 1996). This liver threshold was only exceeded in 5% of samples from red deer from the mined and control areas studied here. Adverse effects on male reproduction have been observed in humans and animals with >300-400 ng/ml of Pb in blood, and changes in spermatogenesis tend to be initiated at a concentration >2 µg/g d.w. in testis, although important differences in Pb accumulation in testis may exist between species (Apostoli et al., 1998). The maximum testis Pb level in red deer from the mining area was 0.110 µg/g d.w. The effects of mining pollution may had been exacerbated by the intense drought occurred in 2005.

Our results indicate that residual mining pollution in the hunting estates of the Sierra Madrona has affected Cu and Se homeostasis and distribution in tissues of red deer. For instance, red deer from the mining area showed higher Cu levels in liver (Reglero et al., 2009), but lower levels in testis than deer from the control sites.

Moreover, levels of Se in liver from mined areas were higher than in control sites (Reglero et al., 29009), although this difference was not observed in testis or in liver of deer used in the present study. The mass of the testis was also higher in deer from mined areas, and this correlated positively with Se and Zn levels. Blottner et al. (1999) in a study on cadmium, also observed a higher mass of testis in roe deer (*Capreolus capreolus*) from a polluted area in Germany (when compared to control areas), and suggested this may be a feedback mechanism whereby more germinative epithelium tissue is generated to compensate for a decrease in the proliferation of spermatogenic cells.

Different forms of SOD are characterized by the transition metal found at their active sites. In eukaryotes, two forms of Cu/Zn SOD are found in the cytosol and in extracellular fluids, and Mn SOD is found in the mitochondria. In rat testis, in addition to cytosolic and mitochondrial SOD, Sertoli cells are capable of synthesizing and secreting extracellular Cu/Zn SOD (Mruk et al., 2002). During spermatozoa transit and storage in the epididymis, the cell undergoes a maturation process where the plasma membrane is modified, and this makes the spermatozoa highly vulnerable to oxidative damage (Vernet et al., 2004). In the epididymis, Cu/Zn SOD and the secreted form of this enzyme catalyse the dismutation of O_2^- to generate H_2O_2. High levels of GPX in the epididymis then convert H_2O_2 into H_2O via the oxidation of GSH into GSSG (Vernet et al., 2004). In the red deer studied here, the SOD activity and the Cu concentration in testis show a positive relationship with spermatozoa membrane viability in the epididymis, which is as we would expect. Eghbali et al. (2008) also observed a positive correlation between Cu levels and SOD activity in seminal plasma of buffalo semen, and rats fed diets low in Cu have shown lower Cu/Zn SOD activity in testis and other tissues (Paynter et al., 1979). Interestingly, we have previously shown that Cu levels in

soil, sediment, plant (Reglero et al., 2008) and deer liver (Reglero et al., 2009) are all higher in the mined areas than in the control areas utilised in this study. Hence, whilst elevated Cu exposure in red deer is demonstrated, testicular Cu is apparently depleted in mined areas when compared to control areas. It is known that high dietary levels of Mo can produce deficiencies in Cu (Johnson et al., 2007) and affect spermiogenesis (Van Niekerk and Van Niekerk, 1989). Since the hepatic Cu levels in deer from the mined areas were higher than those from the controls (Reglero et al., 2009), a Cu imbalance between tissues seems to be occurring in the deer from Sierra Madrona. Alternatively, a lower presence of SOD in testis may have reduced the incorporation of Cu in this tissue, but the evaluation of this hypothesis would need of the study of the expression of SOD in testis.

The other enzyme that could be significantly affected by mining pollution in deer testis and sperm is GPX. This is a selenoenzyme and Se levels are usually positively correlated with the activity of this enzyme when animals have low or normal Se dietary intakes (Lei et al., 1995; Kaushal and Bansal, 2007). In humans, the concentration of Se in seminal plasma is negatively correlated with oxidative damage of DNA in sperm and hence positively correlated with sperm quality (Xu et al., 2003). Nutritional studies have demonstrated the importance of Se for normal male reproduction function, and testis tend to have high concentrations of Se, especially in maturing animals when spermatogenesis begins (Behne et al., 1986). In rats, Lei et al. (1995) found the lowest activity of the classical intracellular form of GPX (GPX1), and the highest activity of the phospholipid hydroperoxide GPX (GPX4) in testis (as compared to other tissues). However, whilst Se status regulates GPX1, it does not specifically regulate GPX4 mRNA levels. Moreover, excess exposure to Se in male rats can actually decrease the GPX4 mRNA expression and reduce GPX activity in testis

and liver (Gan et al., 2002). For the red deer studied here, liver Se levels were not different between the mining and the control areas, but we found evidence in previous studies to suggest that environmental exposure to elevated Se levels was occurring in this particular scenario (Reglero et al., 2008, 2009). Another explanation for the negative correlation we report here for Se against GPX activity and spermatozoa membrane viability may be related to Se speciation. Whilst we have determined total inorganic Se levels here, it is possible that the Se is actually in an organic form which renders it relatively unavailable for GPX synthesis in the testis. As expected, GPX activity was actually positively correlated with spermatozoa membrane viability.

Understanding the speciation of Se in these tissues is important since it plays such a critical role in male reproduction. For example, Se covalently bound to proteins in forms like selenoprotein P (a major form found in plasma) would need to be broken down before the cell could utilize the element (Dumont et al., 2006).

The importance of the antioxidant system in testis to maintain healthy male reproductive function has been highlighted through studying natural processes like aging, or by observing exposure to toxic substances (Tremellen, 2008). Testosterone production by the Leydig cells decreases in aged rats and this is accompanied by a reduction in the activity of SOD and GPX and a decrease in glutathione levels (Luo et al., 2006). Where pollutants are concerned, several transition metals affect the antioxidant system of testis and sperm and hence the sperm quality. Monkeys exposed to chromium show decreased sperm counts and sperm forward motility, and this is accompanied by lower activities of SOD, catalase, and levels of GSH in sperm and seminal plasma in a dose and duration dependent manner (Subramanian et al., 2006). Mice exposed to nickel also exhibited a higher level of lipid peroxidation and DNA damage in testis (Doreswamy et al., 2004). Xu et al. (2003) found positive correlations

between oxidative DNA damage in sperm and Cd in seminal plasma of humans, and negative correlations between these factors and the sperm concentration and viability. However, only a weak correlation was found between Pb in seminal plasma and oxidative DNA damage in sperm. Red deer from the Sierra Madrona mining area are exposed to a mixture of potentially toxic elements, and we have recorded elevated levels of Pb, Cd, Cu and Se in liver, Pb and Se in bone (Reglero et al., 2009), and Pb in testis. The interactions between heavy metals have also been investigated in vitro with salmon sperm. Copper showed a synergistic effect with Cr on oxidative DNA damage, whilst oxidation was diminished by the addition of Cd or Ni (Moriwaki et al., 2008). Moreover, the accumulation of certain essential elements can be affected by overexposure to others (essential or otherwise). A well known example relates to the accumulation of Se in tissues which is triggered by Hg exposure (Rooney, 2007). Although Hg levels in predators from Sierra Morena have been shown to be low Millán et al. (2008), further work should be done on Hg levels in deer to study its possible interaction with Se and male reproductive function.

5. Conclusions.

The study of male sperm function in wildlife offers the opportunity to utilise a novel suite of biomarkers to evaluate exposure to environmental pollutants. In deer from the Sierra Madrona mining area we have observed a significant increase in the mass of the testis in comparison with control animals, and further work will be necessary to study the histopathological changes associated with this tissue proliferation. Moreover, epididymal spermatozoa membrane viability and acrosome integrity were significantly reduced in the deer from the mining area. However, these effects were not directly

associated with observed increases in Pb concentrations in testis or spermatozoa, but were correlated with decreases in the activity of SOD and GPX in testis and spermatozoa. The effects of mining pollution on sperm quality of deer may be related to changes in Cu and Se homeostasis in tissues, since their levels in testis were associated with the observed reductions in SOD and GPX activities, respectively. In the case of Se, contrary to common findings in nutritional studies, levels were negatively associated with GPX activity and spermatozoa viability. Further work is now necessary to identify changes in the expression of these antioxidant enzymes, and to investigate the speciation of Se in order to determine its availability for GPX synthesis in testis.

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CAPÍTULO 2

Changes in fatty acid profiles in testis and spermatozoa of red deer exposed to metal pollution

Mina de Los Pontones (Valle de Alcudia). Foto: Vicente Luchena

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Cambios en la composición de ácidos grasos en testículo y espermatozoide de ciervo común expuesto a contaminación por metales

RESUMEN

En el ciervo común de áreas mineras del Sur-Centro de España se ha observado una disminución en la calidad espermática asociada con la disminución de la actividad de la enzima antioxidante superóxido dismutasa (SOD) en testículo y espermatozoides. También se analizó el perfil de ácidos grasos de espermatozoides y testículos en 29 animales de áreas mineras y se compararon con 33 animales de áreas control. En los ciervos de las áreas mineras, a pesar de la elevada cantidad de Pb en el hígado y en el hueso, las concentraciones de este metal en los espermatozoides y en el testículo no fueron significativamente más elevadas en relación a los animales de las zonas control, pero si se han encontrado diferencias en los niveles de Cu en el testículo que fueron más bajos en los animales de la zona minera. Además en el testículo de animales de estas áreas, ha habido un aumento en el porcentaje de ácido linoleico (18:2n-6) y dihomo-gamma- linolénico (20:3n-6) pero una disminución en la cantidad de ácido araquidónico (20:4n-6) y el porcentaje de este último también fue más bajo en los espermatozoides de los ciervos de zonas mineras. Los niveles de Cu en testículo estuvieron positivamente correlacionados con el porcentaje de ácido araquidónico. Por ello, se piensa que la alteración de la homeostasis del Cu causada por la contaminación por metales puede estar detrás de los efectos observados en los espermatozoides de ciervos de estas áreas.

Palabras clave: reproducción masculina, lípidos, ácido araquidónico, metales pesados, conservación de fauna silvestre.

ABSTRACT

Lowered sperm quality associated with reduced superoxide dismutase activity in testis and spermatozoa has been observed in red deer from a mined area in South-central Spain. Here we present fatty acid profiles for testis and spermatozoa of deer from this mined area (n = 29) and a control area (n=33). Despite elevated Pb in liver and bone of red deer from this area, concentrations in testis and sperm were not significantly higher than in control areas; however, Cu in testis was lower in mined areas. Testis from mined areas also contained higher percentages of linoleic acid (18:2n-6) and dihomo-γ-linolenic acid (20:3n-6), but lower arachidonic acid (20:4n-6). The percentage of 20:4n-6 was also lower in spermatozoa of deer from the mined area. Copper levels in testis correlated positively with the percentage of 20:4n-6. The imbalance in Cu homeostasis caused by metal pollution may have caused the observed effects on deer sperm.

Keywords: male reproduction, lipids, arachidonic acid, heavy metals, wildlife conservation.

1. Introduction.

The Sierra Madrona Mountains and the valley of Alcudia in the province of Ciudad Real, in South-central Spain have been affected by mining and smelting related pollution. This area has some 484 old mines and prospects within an area spanning approximately 2,500 km^2, the majority of which targeted Pb–Zn veins [1]. This zone has been a major Pb producing area in Spain since Roman occupation and up until the late 1900s [2]. The majority of land affected by these mines has never been remediated and the transfer of heavy metals, especially Pb, has been observed to occur via sediments/soils, to plants and then red deer (*Cervus elaphus*). In addition to Pb, higher levels of liver Cd, As and Se were also detected in red deer from this mining area (when compared with nearby control sites [3].

Many metals such as Fe, Cu, Cd, Cr, Co, Pb, Hg, Ni and V have the ability to trigger the generation of reactive oxygen species, resulting in lipid peroxidation, DNA damage, depletion of sulphydryls (SH), and altered calcium homeostasis. Therefore, toxicity associated with these metals may be due (at least in part) to oxidative tissue damage [4]. Whilst Fe, Cu, Cr, V and Co undergo redox-cycling reactions, for a second group of metals (Hg, Cd and Ni) the primary nature of their toxicity relates to the depletion of glutathione (GSH), and to bonds formed with the SH groups of proteins [5]. Metalloids such as As can also induce lipid peroxidation, and one mechanism of action is known to be via the depletion of GSH [5]. Further, Pb can alter antioxidant activities by inhibiting functional SH groups in several enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX) and glucose-6-phosphate dehydrogenase (G6PDH) [6, 7].

In previous work, several biomarkers of oxidative stress were studied in liver, testis and sperm of red deer from the Sierra Madrona-Valle de Alcudia mining area, and some of the effects cited above were observed. For example, red deer from the mined areas had 39% less total GSH (tGSH) in liver than those from control areas [8]. Moreover, red deer from mined areas had lower activities of antioxidant enzymes GPX in testis (-16%), SOD in testis (-14%) and SOD in spermatozoa (-42%) [9]. However, the level of tGSH was 15% higher in testis of red deer from mined areas, and furthermore, deer had larger (9%) and heavier (24%) testis, but lower acrosome integrity (-11%) and membrane viability (-15%) of spermatozoa [9].

Cell damage, as produced by Pb exposure and related oxidative stress, depends largely on the type of fatty acids (FAs) present in cell membranes. Those FAs containing zero to two double bonds are more resistant to oxidative stress than are the polyunsaturated FAs (PUFAs) containing more than two double bonds [6, 10]. Furthermore, changes in FA profiles or composition, especially increases in 20:4n-6 (arachidonic acid), have been linked in several tissues with Pb-exposure in various animal species [11-13]. Other metals, such as Cu and Cd can also affect FA profiles in plants and animals [14, 15]. Spermatozoa contain high levels of PUFA in their membranes [16], and this is functionally important, but this also makes spermatozoa highly susceptible to oxidative damage [17, 18]. Moreover, 20:4n-6 is the precursor compound for eicosanoids such as prostaglandins, which are involved in steroidogenesis in Leydig cells, and in acrosome reactions of spermatozoa [19]. As such, FA profiles in spermatozoa and testis can provide useful information when studying the effects of metals on male reproductive function, both because PUFAs may be susceptible to oxidative damage induced by metals, and FA profiles/metabolism may be altered by these pollutants.

In previous work [8], we observed changes in the FA composition in livers of red deer exposed to metal pollution. In particular, we noted lower percentages of 22:6n-3 (docosahexaenoic acid) in deer from mined areas when compared to control areas. We also observed a negative correlation between Pb concentrations in liver, and the percentage of n-3 PUFA present [8]. The objective herein was to study the FA profiles present in the testis and spermatozoa of red deer, again to detect whether measurable differences existed between mined and control areas, which may then be associated with elevated metal exposure. Such changes in FA composition must be considered if previously observed effects on oxidative stress biomarkers and spermatozoa function are to be correctly interpreted.

2. Methods.

2.1. Study area.

The Sierra Madrona mountains are in the northern part of the Eastern Sierra Morena in the Province of Ciudad Real, in South-central Spain. The elevation here ranges between 500 and 1,300 m. The climate is Mediterranean sub-humid with marked seasons, and Mediterranean shrubland dominates. The most important geological features in this area are a series of major Hercynian WNW–ESE trending anticlines and synclines. The lithostratigraphic sequence is composed of siliciclastic rocks with some interlayered volcanics and rare carbonate levels [1]. The Montes de Ciudad Real region (where most of the the control sites were located) has altitudes between 500 and 1000 m, has similar geomorphological, climatic and biogeographic characteristics to the Sierra Madrona, and is considered to be part of the same natural macroregion [20].

However, this area has not been widely mined historically and is not known to be rich in mineralised zones with high metal contents. As the vegetation in both areas is very similar, we may expect few differences in the antioxidant levels and fatty acid composition in the diet of red deer between sampling sites.

2.2. Sampling.

Nine hunting estates were selected, four of them in mined areas within the Sierra Madrona, and five in control areas in the Montes de Ciudad Real or unmined areas of Sierra Morena. All sampling sites were in the Province of Ciudad Real (Fig. 1).

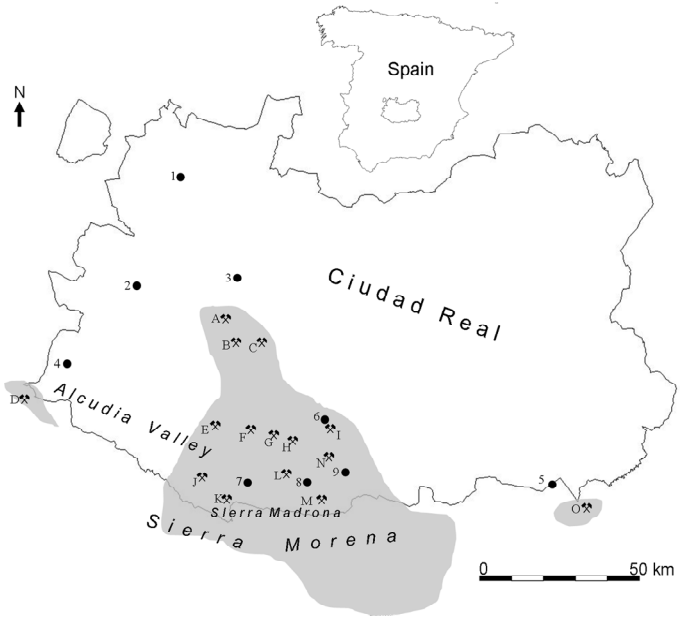

Fig. 1. Study area in the South-central Spanish province of Ciudad Real. The highest concentration of Pb mines is found in the shaded area according to Palero-Fernández and Martín-Izard (2005). Some of the most relevant mines that were close to the sampling sites were Villagutiérrez (A) , La Victoria (B), San Quintín (C), Santa Catalina (D), La Romanilla (E), Cabezarrubias del Puerto (F), Hinojosas de Calatrava (G), Mestanza (H), Las Minillas (I), El Horcajo (J), Fuencaliente (K), Diógenes (L), Los Pontones (M), Los Galayos (N) and Los Engarbes (O). The hunting estates sampled were El Rostro (1), Torneros (2), Hornias Bajas (3), Los Manchones (4), Navalcaballo (5), La Gallega (6), Navalmartina (7), Umbría de Montoro (8) and Tembladera (9).

Samples of 62 adult red deer (>2 years old) were obtained from hunters during autumn 2004. Animal age was precisely assessed from tooth sections of 48 deer [21]. Testes were transported to the laboratory within 1-2 hours of being removed from carcasses, and processed immediately upon arrival. Spermatozoa were recovered from the distal portion of the epididymis by cutting the caudae epididymides with a surgical blade. Oozing sperm mass was collected as described [22], and placed in 1 ml of phosphate saline buffer (pH 7.2; 8 g/l NaCl, 0.2 g/l KCl, 0.1 g/l $MgCl_2 \cdot 6H_20$, 0.1g/l $CaCl_2$, 1 g/l Na_2HPO_4, 0.15 g/l NaH_2PO_4, and 0.2 g/l KH_2PO_4). After spermatozoa collection, an inner portion of the parenchyma of the testis was frozen at -80ºC until analysis for fatty acid (FA) composition.

2.3. Fatty acid composition.

The FA composition was analyzed as described in previous work to evaluate the effect of Pb on lipid metabolism [13]. Testis tissue (0.3 g) or suspension of spermatozoa (200 µl) was added to nine parts (2.7 or 1.8 g) of anhydrous sodium sulphate (Panreac, Barcelona, Spain). After the addition of 10 µl of tridecanoic acid (13:0, 10 µg/µl in methanol; Sigma, Steinheim, Germany) as an internal standard, and 3 or 2 ml of H_2SO_4 1N in methanol (Panreac, Barcelona, Spain), the tube headspace was purged with pure N_2 to prevent oxidation. Tube contents were vortex mixed and then tubes were placed in an oven at 80 ºC for 6 hrs, then at 60 ºC for 12 hrs, whilst undertaking occasional shaking.

FA methyl esters (FAMEs) were extracted with 1.8 or 1.2 ml of *n*-hexane (Panreac, Barcelona, Spain) after adding 3 or 2 ml of deionized water. FAMEs were analyzed by gas chromatography (GC) coupled to an electronic impact-mass

spectrometry detector (EI-MS). The chromatographic system consisted of a 6890N Network GC System with a 5973 Network Mass Selective Detector (Agilent Technologies, Santa Clara, CA, USA). The capillary chromatographic column used was a BPX70 (SGE, 30 m x 0.25 mm I.D., 0.25 μm film thickness; Cromlab, Barcelona, Spain). The injector (splitless mode) was set at 270 ºC and the oven was maintained for 5 min at 100 ºC, then increased to 240 ºC at a ramp rate of 2.5 ºC/min. The carrier gas used was helium at a flow rate of 1.2 ml/min. The mass spectrometer source was held at 230 ºC and the voltage used was 70 volts. Identification and quantification was achieved by comparison of retention times and mass spectra with FAME reference standards (FAMQ-005, AccuStandard, New Haven, CT, USA). Results were expressed as percentages of individual FAMEs from the total amount.

2.4. Elemental analysis.

The concentration of Pb, Cd, Cu, Zn, As and Se were determined in testes and Pb and Se were determined in spermatozoa suspensions. Testis tissues were freeze-dried (Christ Alpha 1-2, Braun Biotech) and dry samples (0.5 g) were digested with 3 ml of HNO_3 (69% Analytical Grade), 1 ml of H_2O_2 (30% v/v Suprapur) and 4 ml of H_2O (Milli-Q grade) using a microwave digestion system (Ethos E, Milestone). Digested samples of testis were diluted to a final volume of 50 ml with Milli-Q H_2O. Spermatozoa suspensions (0.4 ml) were digested in quartz tubes with 2 ml of HNO_3 (65% Suprapur) for 12 h at room temperature. Then, 2 ml of H_2O_2 (30% v/v Suprapur) were added to each tube which was gradually heated in a thermoblock up to 150ºC for 4 h. Digested samples of spermatozoa were diluted to 5 ml with Milli-Q H_2O.

The analysis of As, Se, Cd and Pb was achieved using a graphite furnace atomic absorption spectroscopy system (AAnalyst 800, Perkin Elmer) equipped with an autosampler (AS 800, Perkin Elmer), using 50 µg of $NH_4H_2PO_4$ and 3 µg of $Mg(NO_3)_2$ as matrix modifiers in each atomization for Pb and Cd, and 5 µg of Pd and 3 µg of $Mg(NO_3)_2$ for As and Se. The analyses of Cu and Zn were achieved using a flame atomic absorption spectrometer system (AAnalyst 800 equipped with an autosampler AS 90 plus, Perkin Elmer). Solutions used for calibration were prepared from commercial stock standards with 1 g/l of each element. The limits of detection in testis (LODs, in µg/g dry weight, back-calculated to in-tissue concentrations) were for Pb: 0.03, Cd: 0.002; Zn: 1.5, Cu: 3.7, As: 0.11 and Se: 0.046. The LODs in spermatozoa were 1.45 and 11.3 pg/10^6 cells for Pb and Se, respectively. Blanks were processed in each batch of digestions.

A reference sample of bovine liver (BCR 185R, Community Bureau of Reference) was analyzed (n = 8) and the mean recovery (± SE) was 94.4 ± 5.8% for Pb, 95.8 ± 0.7% for Zn, 100.4 ± 0.6% for Cu, 99.0 ± 4.0% for Cd, 114 ± 11% for As and 74.3 ± 2.2% for Se. A reference sample of pig kidney (BCR 186) was also analyzed in duplicate and the average recovery for Se was 82.6%. All concentrations are given in dry weight (d.w.).

2.5. Statistical analysis.

Data below the detection limits were assigned values of half of the respective LOD for each element for statistical purposes. The concentrations of the elements were log-transformed to attain or approach a normal distribution of the data. Differences in the concentration of the studied elements and the FA compositions in testis and

spermatozoa suspensions between control and mined areas were analysed with Generalized Linear Models (GLMs) taking into account the estate as a nested factor within area (mined and control areas). Deer age was initially included as a factor in these models, but it was removed when it was not significant. A principal component analysis was performed using metal and metalloid levels in testis, and the PC1, PC2 and PC3s calculated were compared between mined and control areas using nested GLMs. Levels of elements in liver and bone (data from [8]), were also compared between areas for these particular males to assess if the same differences previously observed were also seen for this sample of adults.

As the sum of all FAs is always 100, and variations in an individual FA imply changes in the others, effects on the FA profile were studied by means of a compositional analysis [13]. The percentage of each FA was log-transformed, and the ratio with 18:0 (stearic acid) was calculated. A principal component analysis was then performed with the obtained log-ratios and the PC1, PC2 and PC3s calculated were compared between mined and control areas using nested GLMs. The relationships between the principal components for the elements analysed and the FA profiles, and the FAs and the elements differing between areas were studied by linear correlations. Analyses of covariance were used to determine if correlations were explained by linear relationships between elements and FAs, or, due to differences between areas. The criterion for significance was set at $p \leq 0.05$. Statistics were performed with the SPSS 15.0 program.

3. Results.

Deer from mined areas had significantly lower concentrations of Cu in testis (43.3% less, $p<0.001$) than deer from control areas (Table 1). The levels of all other elements in testis and spermatozoa did not differ between the two study areas. The PCA on the element concentration data for testis showed that the three components (PC1, PC2 and PC3) accounted for 73% of the observed variance, with 38%, 18% and 17% accounted for respectively. PC1 also correlated positively with Cu, Zn and Se levels in testis, PC2 with As, and PC3 with Pb. PC1 data differed significantly between the control and mined areas ($p = 0.026$) and PC2 data was almost significantly different by area ($p = 0.055$, Fig. 2). Higher metal exposure in deer from the mined area was shown for Pb in liver where the mean and 95% CIs were 0.44 (0.35-0.56) *vs.* 0.18 (0.11-0.29) µg/g d.w., ($p < 0.001$), and in bone were 1.32 (1.07-1.63) *vs.* 0.94 (0.75-1.17) µg/g d.w., ($p < 0.001$). Deer from the mined area also had more Cu in liver than in the control area, with 71 (59-86) *vs.* 52 (44-62) µg/g d.w., ($p = 0.011$), more Se in liver with 0.25 (0.20-0.31) *vs.* 0.13 (0.10-0.16) µg/g d.w., ($p < 0.001$), more Se in bone with 0.26 (0.22-0.30) *vs.* 0.23 (0.20-0.26) µg/g d.w., ($p = 0.022$) and less As in bone at 0.22 (0.19-0.24) *vs.* 0.19 (0.17-0.21) µg/g d.w., ($p = 0.004$). All other element concentrations in liver and bone did not differ between areas.

Table 1. Geometric mean, 95% confidence intervals and range of concentrations of metals and metalloids in testis and spermatozoa of red deer residing in historically mined (polluted) and unmined (control) areas.

	Element	Area	n	Mean	95% CI	Range
Testis	Pb	Control	33	0.028	0.024-0.034	ND-0.059
(µg/g dw)		Mines	29	0.027	0.020-0.037	ND-0.571
	Cu	Control	33	10.3	9.1-11.7	4.3-15.8
		Mines	29	5.9*	5.1-6.8	*ND*-13.5
	Zn	Control	33	95.2	93.8-96.6	84.0-102.3
		Mines	29	81.3	61.7-107.2	1.9-99.6
	As	Control	33	0.056	0.053-0.060	ND-0.136
		Mines	29	ND	ND	ND
	Se	Control	33	1.08	1.04-1.13	0.79-1.30
		Mines	29	0.93	0.76-1.14	ND-1.53
	Cd	Control	33	0.021	0.018-0.025	0.008-0.039
		Mines	29	0.015	0.012-0.019	0.008-0.042
Spermatozoa	Pb	Control	13	ND	ND	ND
(pg/10^6 spz)		Mines	19	1.57	1.33-1.84	ND-6.19
	Se	Control	13	99.7	74.3-133.7	46.1-195.5
		Mines	19	120.0	88.8-162.3	53.5-1104.4

*Nested GLM, $p<0.001$, ND = not detected

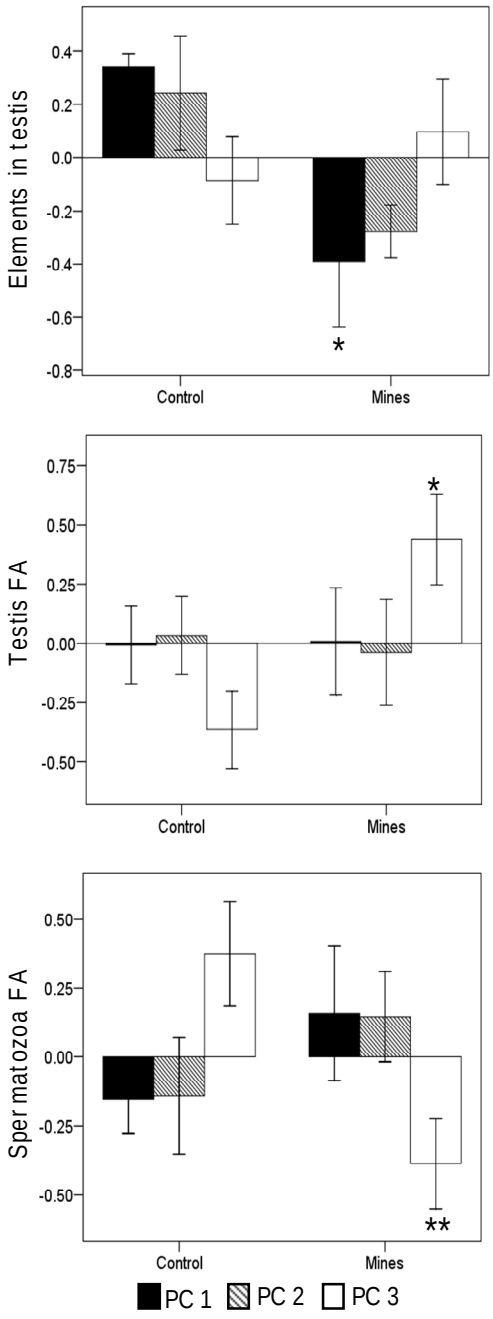

Fig. 2. Differences between the control and mined areas in terms of the first three principal components (PC1-3) extracted from the principal component analyses (PCA) of element concentrations in testis (top), fatty acid (FA) composition in testis (middle), and FA composition in epididymal spermatozoa (bottom) of red deer. Means ± standard error are given. GLM: $p^* \leq 0.05$, $p^{**} \leq 0.001$.

Testis of red deer from the mined areas contained higher percentages of two n-6 PUFAs (18:2n-6 (linoleic acid) and 20:3n-6 (dihomo-γ-linolenic acid)), two saturated FAs (14:0 (myristic acid) and 16:0 (palmitic acid)) and the total saturated FA than those from control areas (Table 2). On the contrary, percentages of 20:4n-6 and 20:3n-9 (Mead acid) were lower in the mined area. The PCA for the FA profiles in testis showed that seven components accounted for 76% of the observed variance. PC1, PC2 and PC3 accounted for 26%, 17% and 11% of the observed variance respectively. PC1 correlated positively with n-3 PUFA in testis, PC2 with monounsaturated FA, and PC3 with 18:2n-6. Only PC3 data differed significantly between control and mined areas ($p = 0.025$; Fig. 2).

Red deer epididymal spermatozoa from mined areas contained a lower percentage of 20:4n-6 (50% less) than in control areas, and consequently, the n-3/n-6 PUFA ratio tended to be higher in the mined areas (Table 3). The percentages of 18:0 and 18:1n-9 (oleic acid) were also lower in mined areas, and the percentage of total PUFA tended to be higher. The PCA for the FA composition in spermatozoa showed that 13 components accounted for 77% of the observed variance. PC1, PC2 and PC3 only accounted for 11%, 9% and 8% of the variance respectively. PC1 correlated positively with n-6 PUFA in spermatozoa, PC2 with monounsaturated FA and 18:3n-6 (γ-linolenic acid), and PC3 correlated with monounsaturated FA and 20:4n-6. Only the PC3 data differed significantly between the control and mined areas ($p = 0.001$; Fig. 2).

Table 2. Mean (± standard error) fatty acid (FA) composition (%) in testis of red deer residing in historically mined (polluted) and unmined (control) areas.

	Control (n = 30)	Mined (n = 26)	GLM
	Mean ± SE	Mean ± SE	p
14:0	0.09 ± 0.00	0.10 ± 0.01	0.044
15:0	0.05 ± 0.00	0.05 ± 0.00	*ns*
DMAMD	3.23 ± 0.06	3.21 ± 0.07	*ns*
16:0	19.48 ± 0.23	20.31 ± 0.21	0.001
16:1n-9	0.35 ± 0.01	0.34 ± 0.02	*ns*
16:1n-7	0.33 ± 0.02	0.30 ± 0.01	*ns*
M16:0 I	0.04 ± 0.00	0.05 ± 0.00	*ns*
M16:0 A	0.03 ± 0.00	0.03 ± 0.00	*ns*
17:0	0.15 ± 0.01	0.18 ± 0.01	*ns*
DMAOD	0.51 ± 0.02	0.49 ± 0.02	*ns*
18:0	6.84 ± 0.10	6.75 ± 0.17	*ns*
18:1n-9	11.89 ± 0.22	11.50 ± 0.21	*ns*
18:1n-7	2.69 ± 0.08	2.73 ± 0.09	*ns*
18:2n-6	2.58 ± 0.12	3.24 ± 0.15	0.003
19:0	<0.01 ± 0.00	<0.01 ± 0.00	*ns*
18:3n-6	0.01 ± 0.00	0.01 ± 0.00	*ns*
18:3n-3	<0.01 ± 0.00	0.01 ± 0.00	*ns*
20:0	0.03 ± 0.00	0.03 ± 0.00	*ns*
20:3n-9	0.15 ± 0.01	0.09 ± 0.01	0.011
20:2n-6	<0.01 ± 0.00	<0.01 ± 0.00	*ns*
20:3n-6	1.88 ± 0.08	2.15 ± 0.08	0.008
20:3n-3	0.02 ± 0.01	0.02 ± 0.01	*ns*
22:0	0.26 ± 0.01	0.24 ± 0.01	*ns*
20:4n-6	18.65 ± 0.27	17.73 ± 0.39	0.008
23:0	0.03 ± 0.00	0.02 ± 0.00	*ns*
22:4n-6	0.30 ± 0.02	0.30 ± 0.03	*ns*
22:5n-6	0.32 ± 0.06	0.24 ± 0.03	*ns*
24:0	0.19 ± 0.01	0.18 ± 0.01	*ns*
22:5n-3	0.82 ± 0.06	0.86 ± 0.08	*ns*
22:6n-3	29.09 ± 0.43	28.85 ± 0.63	*ns*
SAT	27.19 ± 0.21	27.93 ± 0.22	0.004
MONO	15.25 ± 0.27	14.88 ± 0.25	*ns*
n-6 PUFA	23.74 ± 0.28	23.66 ± 0.33	*ns*
n-3 PUFA	29.93 ± 0.44	29.73 ± 0.65	*ns*
PUFA	53.68 ± 0.41	53.40 ± 0.43	*ns*
n-3/n-6 PUFA	1.27 ± 0.03	1.27 ± 0.04	*ns*

ns = not significantly different by area using a nested generalized linear model (GLM).

Table 3. Mean (± standard error) fatty acid (FA) composition (%) in epididymal spermatozoa of red deer residing in historically mined (polluted) and unmined (control) areas.

	Control (n = 28)	Mined (n = 27)	GLM
	Mean ± SE	Mean ± SE	*p*
14:0	8.03 ± 0.39	7.97 ± 0.50	*ns*
15:0	0.17 ± 0.14	0.03 ± 0.02	*ns*
DMAMD	10.49 ± 0.88	10.67 ± 0.65	*ns*
16:0	18.42 ± 0.97	16.50 ± 1.02	*ns*
16:1n-9	0.14 ± 0.09	0.05 ± 0.02	*ns*
16:1n-7	0.24 ± 0.21	0.03 ± 0.01	*ns*
M16:0 I	0.11 ± 0.10	0.06 ± 0.03	*ns*
M16:0 A	0.07 ± 0.03	0.04 ± 0.03	*ns*
17:0	0.25 ± 0.14	0.02 ± 0.01	*ns*
DMAOD	0.09 ± 0.04	0.03 ± 0.02	*ns*
18:0	6.29 ± 0.25	4.59 ± 0.27	<0.001
18:1n-9	0.54 ± 0.11	0.27 ± 0.04	0.022
18:1n-7	0.36 ± 0.06	0.29 ± 0.07	*ns*
18:2n-6	0.43 ± 0.08	0.40 ± 0.09	*ns*
19:0	0.02 ± 0.02	0.08 ± 0.06	*ns*
18:3n-6	0.09 ± 0.05	0.08 ± 0.03	*ns*
18:3n-3	0.28 ± 0.15	0.03 ± 0.02	*ns*
20:0	0.08 ± 0.02	0.12 ± 0.05	*ns*
20:3n-9	0.05 ± 0.03	0.06 ± 0.04	*ns*
20:2n-6	1.09 ± 0.63	0.06 ± 0.04	*ns*
20:3n-6	0.10 ± 0.05	0.22 ± 0.08	*ns*
20:3n-3	0.39 ± 0.16	0.20 ± 0.15	*ns*
22:0	0.15 ± 0.05	0.20 ± 0.07	*ns*
20:4n-6	3.95 ± 0.48	1.98 ± 0.33	0.004
23:0	0.08 ± 0.05	0.09 ± 0.06	*ns*
22:4n-6	0.32 ± 0.12	3.00 ± 2.84	*ns*
22:5n-6	0.25 ± 0.12	0.20 ± 0.08	*ns*
24:0	0.12 ± 0.07	0.28 ± 0.19	*ns*
22:5n-3	0.18 ± 0.05	0.55 ± 0.21	*ns*
22:6n-3	47.22 ± 1.64	51.87 ± 2.45	*ns*
SAT	33.79 ± 1.29	30.00 ± 1.60	*ns*
MONO	1.28 ± 0.32	0.64 ± 0.09	*ns*
n-6 PUFA	6.23 ± 1.00	5.95 ± 2.86	*ns*
n-3 PUFA	48.06 ± 1.48	52.65 ± 2.34	*ns*
PUFA	54.29 ± 1.35	58.60 ± 1.55	0.057
n-3/n-6 PUFA	12.06 ± 1.72	28.08 ± 7.82	0.068

ns = not significantly different by area using a nested generalized linear model (GLM).

DMAHD: dimethyl acetal of hexadecanal, DMAOD: dimethyl acetal of octadecanal, SAT: saturated FA, MONO: monounsaturated FA

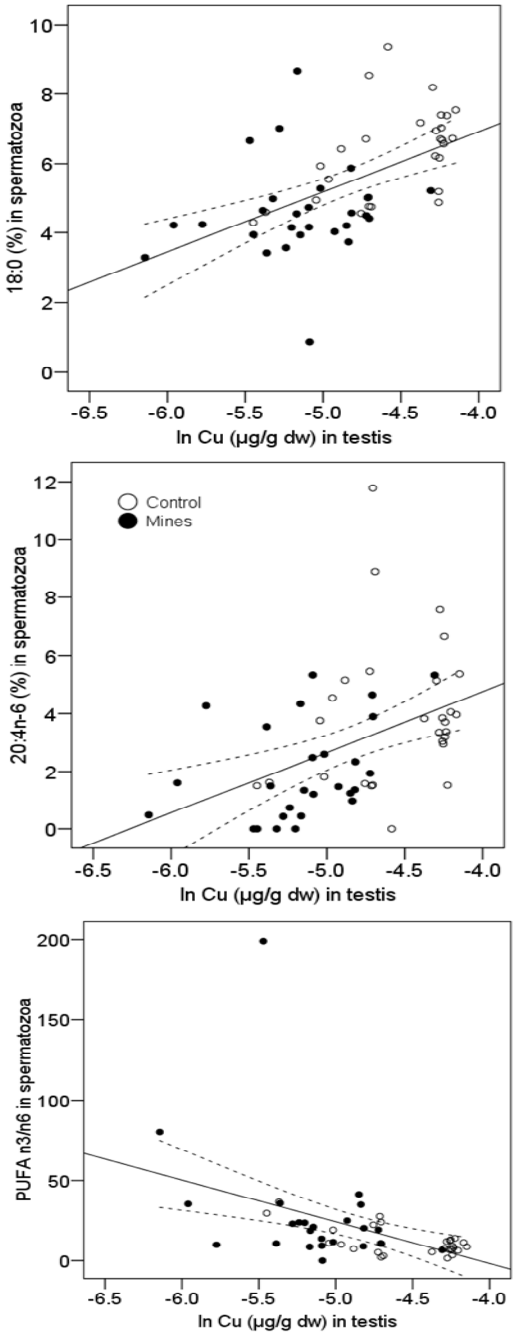

Fig.3. Relationships between natural-log transformed Cu levels in testis and the percentage of 18:0 (stearic acid) ($r = 0.534$, $p<0.001$; top), 20:4n-6 (arachidonic acid) ($r = 0.436$, $p = 0.001$; middle) and n-3/n-6 PUFA ratio ($r = -0.444$, $p=0.001$; bottom) in epididymal spermatozoa of red deer from control (white dots) and mined areas (black dots).

The most significant correlations between the FA data and those elements that differed between control and mined areas were observed for Cu in testis and the percentages in spermatozoa of 18:0, 20:4n-6, and the n-3/n-6 PUFA ratio (Fig. 3). An ANCOVA showed for the percentage of 18:0 in spermatozoa, area had a significant effect (lower percentages were seen in mined areas, $p = 0.020$) and that Cu levels in testis were positively related ($p = 0.027$). However, Cu levels in testis were only marginally associated with 20:4n-6 percentages in spermatozoa ($p = 0.074$). The n-3/n-6 PUFA ratio in spermatozoa was inversely associated with Cu in testis ($p = 0.010$) but was not affected by area (mined/control). Finally, neither metal levels nor FA compositions in testis and spermatozoa were affected by deer age, so it was not included in the final nested GLMs.

4. Discussion.

Lead levels in testis and spermatozoa were not significantly different between the control and mined areas, as was the case in deer hunted in 2005 in the same area. However, an imbalance in certain elements may have been produced by the metal pollution present in mined areas because Cu levels were significantly lower in testis of the deer from the contaminated zones. This reduced level of Cu in testis was also observed by Reglero et al. [9]. Therein, Cu levels in testis were also negatively correlated with Pb, which may have indicated an interaction was occurring between these metals. Moreover, testis Cu levels were positively correlated with SOD activity in testis and membrane viability of spermatozoa, which were both in turn lower in mined areas [9]. Here, we have continued to observe an effect of the metal pollution present on the regulation of Cu levels in testis. Furthermore, we show that changes in the FA

profiles of spermatozoa were associated with Cu levels, and this was reflected by positive correlations between essential Cu and percentages of 18:0 and 20:4n-6, and a negative correlation with n-3/n-6 PUFA ratios. These results indicate that a possible mechanism of action of potentially toxic metals on male reproductive function in deer is mediated in some way by an altered regulation of Cu in testis.

The changes in FA composition in testis of red deer from mined areas that we observed predominantly involved increases in saturated FAs (mainly 16:0) and two n-6 PUFAs, i.e., 18:2n-6 and 20:3n-6. However, the next step in PUFA synthesis, i.e., the formation of 20:4n-6, was not enhanced, in fact, there was a decrease in this FA.

In spermatozoa, the main changes we observed in deer from mined areas were decreases in 18:0, 18:1n-9 and 20:4n-6, but, we did not note changes in the precursor to the last of these, i.e., 18:2n-6. In fact, total PUFA was noted to increase in deer spermatozoa from mined areas. It is important to say that percentages of 22:6n-3, a major FA in testis and semen of many mammal species, including humans [23, 24], did not differ between areas. The synthesis pathway of n-3 PUFA (18:3n-3 → 18:4n-3 → 20:4n3 → 20:5n-3 → 22:5n-3 → 24:5n-3 → 24:6n-3 → 22:6n3 [25]) seems to have been less affected by metal exposure, than the n-6 pathway.

The n-6 PUFA synthesis pathway in vertebrates normally involves the conversion of dietary 18:2n-6 to 18:3n-6 by Δ6 desaturase, the elongation to 20:3n-6, and then the conversion to 20:4n-6 by Δ5 desaturase [25]. In testis, Sertoli cells are the most important cells for the metabolism of essential FAs, and germ cells are dependent on a supply from these cells [26]. Here, in deer testis from mined areas we observed a slight decrease in 20:4n-6, accompanied by an increase in its precursors (both 18:2n-6 and 20:3n-6). This accumulation of substrate may indicate that a decrease in the activity of Δ5 desaturase was occurring in the testis of deer from mined areas. Moreover,

suppressed levels of 20:4n-6 were more evident in spermatozoa, and this was associated with reduced Cu levels in deer testis. In previous work, Cu deficiency has been associated with a decreased activity of desaturases in liver microsomes [27], however, chronic Cu exposure in aquatic invertebrates has caused increases in long-chained PUFA percentages [15]. Several studies with rat, mouse and humans indicates that inadequate Cu intake impairs the ability to monounsaturate long chain saturated fatty acids, and that conversely, Cu supplementation often causes increases in monounsaturated fatty acids [28]. Among other changes in FA profiles, Cu deficiency also reduced 20:4n-6 contents in mice liver [29], but caused increased levels in human plasma [30]. High dietary levels of Mo in domestic ruminants induce Cu deficiency, produce testicular degeneration and affect spermiogenesis in ram [31-33]. Tetrathiomolybdate used as an antiangiogenic agent for cancer therapy produces in rats reduced epididymal weights, sperm counts and sperm motility, that can be prevented by dietary Cu supplementation [34]. In humans, Mo levels in blood have also been inversely associated with Cu levels and sperm quality [35]. Further research in our study area should be directed in the future to study the interaction between Mo and Cu in red deer.

As noted here, in deer testis, Mateo et al. [13] found increases in saturated FAs and 18:2n-6 percentages in livers of mallard ducks exposed to Pb. However, therein 20:4n-6 was also elevated in Pb exposed animals, whilst here we observed (in testis and spermatozoa) decreased 20:4n-6 in deer from the mined areas. In accordance with Mateo et al. [13], other studies have also noted increases in percentages of 20:4n-6, or, in the ratio between 20:4n-6 and 18:2n-6 in tissues of animals exposed to Pb. For example, Knowles and Donaldson [36] found 20:4n-6 levels in liver doubled when broiler chicks (*Gallus domesticus*) were exposed to 1500 ppm Pb in diet. Similar results

were obtained when turkey poults (*Meleagris gallopavo*) were fed with 100 ppm of dietary Pb [37]. Dorman and Freeman [38] also observed an increased release of 20:4n-6 by vascular smooth muscle cell cultures in the presence of 100 µM Pb acetate. Increases were also seen in blood cells from humans occupationally exposed to Pb [12]. Differences observed between our field and these experimental studies may be attributable to the complex mixture of metals and metalloids to which deer are exposed in the field (in mined areas) when compared to laboratory studies based on a single toxic element.

The mechanism by which changes in FA profiles are driven by exposure to other metals (apart from Cu and Pb) also seems to be related to perturbations of the FA desaturation process [39]. In an experimental study [40], rat hepatocytes were cultured with $CdCl_2$, and showed a concentration-dependent reduction in the conversion of 18:0 to 18:1n-9, and a slight increase in the rate of conversion of 18:2n-6 to 20:4n-6. The first of these conversion steps is caused by a membrane bound 18:0-CoA ($\Delta 9$) desaturase. The second is likely to be catalyzed by a $\Delta 6$ desaturase which uses dietary 18:2n-2 as a substrate (since mammals lack $\Delta 12$ desaturases to convert 18:1n-9 to 18:2n-6 [25]). These Cd related effects were probably mediated by changes in the expression of these enzymes because Cd did not inhibit liver microsomal $\Delta 9$ desaturase [41].

The decreases in 20:4n-6 we observed here in spermatozoa may have consequences on the fluidity of the cell membrane, and this may explain the reduced membrane viability we observed previously [9]. In human leukaemia T-cells cultured under Zn deficient conditions, Verstraeten et al. [42] observed a decrease in 20:4n-6, higher membrane fluidity in the hydrophobic region of the bilayer, and an associated higher frequency of apoptosis. Argov et al. [43] also found lower bovine semen quality

in the summer, when the contents of 20:4n-6 and 18:2n-6 in spermatozoa membranes were also lower. The FA profile in human sperm has also been noted to affect spermatozoa motility and male fertility. Asthenozoospermic samples have shown lower levels of certain PUFAs (such as 20:4n-6 and 22:6n-3) and higher amounts of some saturated FAs (14:0, 16:0 and 18:0) than have normozoospermic samples [44]. Further, spermatozoa from oligozoospermic men had lower levels of 22:6n-3 than those from normozoospermic men [45].

5. Conclusions.

Despite current knowledge regarding the adverse effects of Pb and other heavy metals on male reproductive function [46-48], surprisingly few studies have been reported in wildlife [49, 50]. In a previous study with red deer from the Sierra Madrona-Valle de Alcudia mining area, we observed several significant metal exposure related differences in the size of testis, and several measures of sperm quality (in comparison with control areas [9]). Therein, the most relevant change in the concentration of metals in the testis was an observed decrease in Cu, and this has been also observed with different individuals here as well. We have also observed a positive relationship between the concentration of Cu in testis and 20:4n-6 content in deer spermatozoa. Reglero et al. [9] further observed decreased membrane viability in deer spermatozoa from mined areas, and this was associated with lowered SOD activity and hence an increased risk of suffering oxidative stress. Possibly, since Cu is a key component in this enzyme, a positive correlation between SOD activity in spermatozoa and Cu level in testis was expected [9]. Moreover, the reduced sperm membrane viability previously observed may now be explained by observations made here, i.e., where lowered level of

20:4n-6 was noted, which has been linked to reduced fertility in men. The reduction in the presence of 20:4n-6 in spermatozoa we observed may have caused some of the alterations in the sperm quality of the deer from this polluted area noted previously [9]. This may have occurred either because this compound is a key precursor in the synthesis of prostaglandins (which are involved in spermatozoa capacitation), or, because reduced levels of 20:4n-6 may have affected membrane fluidity.

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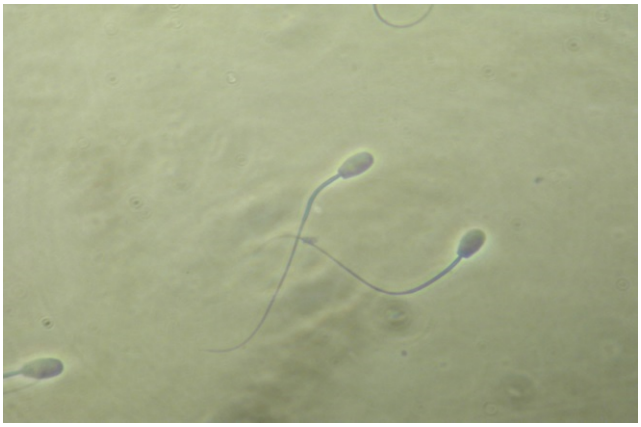
CAPÍTULO 3

Increased chromatin fragmentation and reduced acrosome integrity in spermatozoa of red deer from a lead mining areas

Espermatozoides con cromatina intacta y alterada (Microfotografía de fluorescencia)



Microfotografía de espermatozoides sin acrosoma íntegro. Foto: Pilar Castellanos

Castellanos P, del Olmo E, Fernández-Santos MR, Rodríguez-Estival J, Garde JJ, Mateo R**.** 2014. (*Submitted*).

Incremento de fragmentación de la cromatina y reducción de la integridad del acrosoma en espermatozoide de ciervo común expuesto a contaminación por plomo

RESUMEN

Los vertebrados están expuestos constantemente a la contaminación difusa por metales pesados presentes en el ambiente, pero en algunos casos la proximidad a fuentes de emisión como es la actividad minera aumenta el riesgo de desarrollar los efectos adversos de estos contaminantes. En este estudio, hemos analizado en ciervos comunes (*Cervus elaphus*) de áreas mineras (n=37) en relación a otros animales de áreas control (n= 26), los niveles de plomo (Pb^{2+}) en los espermatozoides y testículo, los daños en la cromatina así como la actividad de antioxidantes endógenos, como la glutatión peroxidasa (GPX) en los espermatozoides para analizar los efectos de este metal.

Los ciervos de la zona contaminada por Pb presentan mayores niveles de este metal tanto en el parénquima y la cola del epidídimo, como en los espermatozoides junto a un porcentaje inferior de espermatozoides con acrosoma intacto. También ha habido un aumento en la actividad de la glutatión peroxidasa y en los índices de fragmentación de la cromatina (XDFi) y de cromatina inmadura o anormalmente condensada (HDS) en los ciervos de áreas mineras en relación a las zonas control. Estos datos muestran que la contaminación en las zonas mineras puede producir en la fauna silvestre daños en la cromatina y en la membrana de los espermatozoides.

Palabras clave: *c*alidad espermática, metales pesados, daños en el ADN, SCSA, fauna silvestre.

ABSTRACT

Vertebrates are constantly exposed to a diffuse pollution of heavy metals existing in the environment, but in some cases, the proximity to emission sources like mining activity increases the risk of developing adverse effects of these pollutants. Here we have studied lead (Pb) levels in spermatozoa and testis, and chromatin damage and levels of endogenous antioxidant activity in spermatozoa of red deer (*Cervus elaphus*) from a Pb mining area (n=37) and a control area (n=26). Deer from the Pb-polluted area showed higher Pb levels in testis parenchyma, epididymal cauda and spermatozoa, lower values of acrosome integrity, higher activity of glutathione peroxidase (GPx) and higher values of DNA fragmentation (X-DFI) and stainability (HDS) in sperm than in the control area. These results indicate that mining pollution can produce damage on chromatin and membrane spermatozoa in wildlife.

***Keywords*:** sperm quality, heavy metals, DNA damage, SCSA, oxidative stress, wildlife.

1. Introduction.

Lead (Pb) is a heavy metal widely present in the environment because of its use by man for centuries. Mining and smelting areas show elevated levels of Pb pollution during and beyond their production period (Wilson and Pyatt, 2007; Beyer et al., 2007). One of these polluted sites is the Alcudia Valley – Sierra Madrona Pb-Zn mining area, where argentiferous galena was extracted since the Roman expansion in the Iberian Peninsula until the 2nd half of the 20th century, when the last mines closed. Nowadays, water, soil and plants around these mines are Pb polluted (Reglero et al., 2008); and this affects wildlife and livestock by altering their health status and biological functions such as reproduction (Reglero et al., 2009a, 2009b; Rodríguez-Estival et al., 2011, 2013).

Heavy metals can produce adverse effects on animals, including the reduction of sperm quality (Lavranos et al., 2012; Marzec-Wroblewska et al., 2012), through several mechanism of action such as competing with other cations, binding with sulfhydryl groups of proteins or depleting GSH levels (Jornova and Valko, 2011). In the case of Pb, changes produced on oxidative stress parameters can vary between tissues, especially between reproductive and others tissues (Reglero et al., 2009a, 2009b; Tvrda et al., 2013). An example of these differences in the response to oxidative stress among tissues has been observed in red deer (*Cervus elaphus*) from Alcudia Valley – Sierra Madrona mining area. Red deer from the mined area had 39-56% less total GSH (tGSH) in liver than those from control sites (Reglero et al., 2009a; Rodríguez Estival et al., 2011); and a similar reduction (28-46%) was found in spleen (Rodríguez-Estival et al., 2013). On the contrary, tGSH level in testis was 15% higher in the mining area than in the control sites (Reglero et al., 2009b). Moreover, red deer from the mining area had

higher activities of SOD in liver (98%; Rodríguez-Estival et al., 2011) and GPX in spleen (81-130%; Rodríguez-Estival et al., 2013) than in the control sites. The effect of metal pollution was again different in the reproductive system, because lower activities of GPX (-16%) and SOD (-14%) were found in testis, as well as lower activity of SOD in spermatozoa (-42%) (Reglero et al., 2009b). Testis and spermatozoa of red deer from the mining area also showed lower arachidonic acid (20:4n-6) percentages in the fatty acid composition than in the control area (Castellanos et al., 2010). The lower SOD activity found in testis was associated with a reduction in the Cu levels in testis of deer from the mining area (Reglero et al., 2009b). The percentage of 20:4n-6 in spermatozoa was also positively correlated with Cu levels in testis (Castellanos et al., 2010). These results suggested that Pb pollution in the mining area altered Cu homeostasis and this had consequences on the activity of cytosolic and extracellular Cu/Zn SOD. Arachidonic acid is important in sperm function, because it is released from spermatozoa membranes by phospholipase A to be used by lipoxygenase for producing 15-hydroxyl-5,8,11,13-eicosatetraenoic acid (15-HETE) and prostaglandins (PGE) involved in the process of acrosome reaction and sperm capacitation (Breitbart and Spungin, 1997). These changes may explain the lower acrosome integrity and sperm viability found in red deer from Alcudia Valley – Sierra Madrona Pb mining area (Reglero et al., 2009b).

Oxidative DNA damage has been associated with lowered fertility in several species (Aitken and Sawyer, 2003; Gillan et al., 2005). Pb exposure in humans has been associated with oxidative damage of DNA and alteration of the chromatin stability in spermatozoa (Quintanilla-Vega et al., 2000; Xu et al., 2003). In the present study, we have tested the hypothesis that red deer from Alcudia Valley – Sierra Madrona mining area could have higher levels of DNA damage than deer from control sites. The sperm

chromatin structure assay (SCSA) has been used in our study to detect DNA damage and/or alterations in the histones and protamines of nucleosomes (Evenson, 2013), and to analyze the correlation between genotoxic effect of Pb exposure, the quality parameters of sperm and their response to oxidative stress caused by this metal.

2. Material and methods.

2.1. Study area.

The study area extends along the Alcudia Valley – Sierra Madrona mining area and a control area at Montes de Ciudad Real region (Central Spain). Further details of these study areas have been described in Reglero et al. (2009a).

2.2. Sampling.

Testes of 63 adult red deer (*Cervus elaphus*) were obtained from hunters during the autumns of 2008 and 2009 in the mining (n = 37) and the control areas (n = 26). The samples were collected after ‘‘monterias’’ (large driven hunts) in five hunting estates of the mining area and another five estates from the control area (Fig.1). The testes were transported in a portable cooler to the laboratory within 1–2 h of being removed from the carcasses. Then, spermatozoa were collected from cauda epididymides as described by Soler et al. (2005). Briefly, spermatozoa were obtained by cutting the distal portion of the epididymis with a surgical blade and were placed in 1 ml of phosphate saline buffer (pH 7.2; 8 g/l NaCl, 0.2 g/l KCl, 0.1 g/l $MgCl_2$. $6H_20$, 0.1 g/ l $CaCl_2$, 1 g/l Na_2HPO_4, 0.15 g/l NaH_2PO_4, and 0.2 g/l KH_2PO_4) containing bovine serum albumin (at

5 g/l). After sperm collection, motility, viabilility and acrosome integrity were evaluated. Moreover, sperm chromatin stability was assessed. The remaining sperm sample was frozen at -80 ºC for the determination of oxidative stress biomarkers and Pb analysis. Testes were then frozen at -80 ºC for further analysis of Pb levels in testicular parenchyma and epididymal cauda tissue.

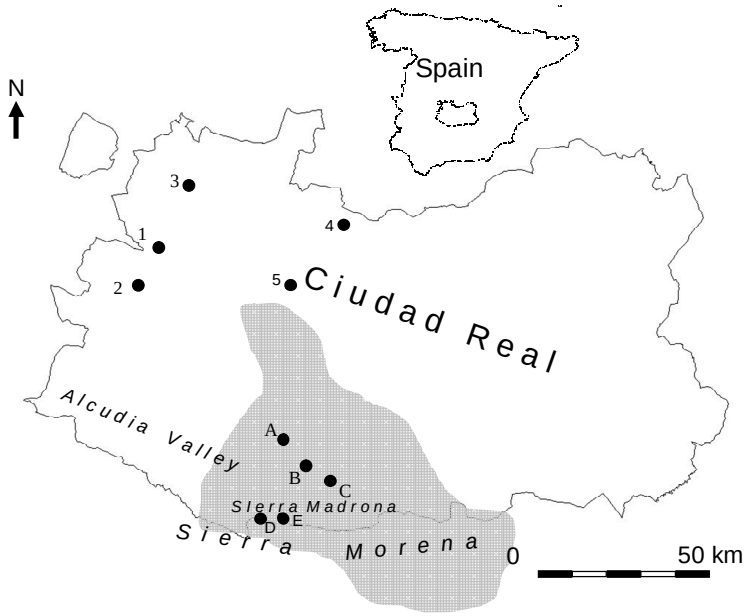


Fig.1. Study area in the province of Ciudad Real. Pb mines are found in the shaded area. Hunting states in the mining areas were Zamorillas (A), El Acebuchar (B), El Castaño (C), Chorreras (D), Navalmartina (E); hunting states in the control area were Puebla de Don Rodrigo (1), Torneros (2), Rio Frío (3), Quintos de Mora (4), Ballesteros (5).

2.3. Sperm quality measurements.

After spermatozoa collection, a routine sperm evaluation was carried out. Sperm concentration, sperm viability and acrosome integrity were studied following the protocols described by Soler et al. (2005). Briefly, sperm concentration in the suspension was determined by microscopy in a Bürker counting chamber. The

percentage individual motile sperm (motility) was noted, and the quality of motility was assessed using a scale that ranged from 0 (lowest, immobile or dead) to 5 (highest, progressive and vigorous movement). Membrane integrity was evaluated by using a nigrosin-eosin stain (NE). The NE stain was prepared as described by Tamuli and Watson (1994). The diluted sperm (5 µl) was mixed with the NE stain (10 µl) at 37 °C, incubated for 30 sec, smeared and dried on a warm plate at 37 °C. The samples were evaluated using bright field microscopy at ×400. Live spermatozoa remained unstained, while dead cells were dull pink. The % of live spermatozoa was expressed as viability. Acrosomal integrity was evaluated after a 1:10 dilution in 2% glutaraldehyde in 0.165M cacodylate/HCl buffer (pH 7.3). The percentage of spermatozoa with intact acrosomes (i.e., with normal apical ridges) was assessed at ×400 under phase-contrast optics.

2.4. Analysis of oxidative stress biomarkers.

Sperm suspensions were adjusted to 240 × 10^6 spermatozoa/ml in 0.5 ml of buffer before antioxidants analysis. Levels of total glutathione (tGSH) and the activities of glutathione peroxidase (GPx; EC 1.11.1.9) and superoxide dismutase (SOD; EC 1.15.1.1) were measured following the micromethods described by Reglero et al. (2009a) adapted to an automated spectrophotometer (A25-Autoanalyzer; BioSystems) at 37 ºC. These three oxidative stress parameters were selected because their values were found to differ between the mining and the control area in a previous study with deer sperm and testis (Reglero et al., 2009b).

2.5. Sperm chromatin structure assay (SCSA).

Chromatin stability was assessed following the Sperm Chromatin Structure assay (SCSA®), based on the susceptibility of sperm DNA to acid-induced denaturation in situ and on the subsequent staining with the metachromatic fluorescent dye of acridine orange (Evenson and Jost 1994, 2013). Acridine orange fluorescence shifts from green (dsDNA; double strand) to red (ssDNA; single strand) depending on the degree of DNA denaturation. Samples were diluted in TNE buffer (0.01 M Tris–HCl, 0.15 M NaCl, 1 mM EDTA, pH 7.4) to a final sperm concentration of 2×10^6 cells/ml. Acid-induced denaturation of DNA in situ was achieved by adding 0.4 ml of an acid-detergent solution (0.17% Triton X-100, 0.15 M NaCl, 0.08 N HCl, pH 1.4) to 200 µl of sample. After 30 s, the cells were stained by adding 1.2 ml of an acridine orange solution (0.1 M citric acid, 0.2 M Na2HPO4, 1 mM EDTA, 0.15 M NaCl, 6 µg/ml acridine orange pH 6.0). The stained samples were analyzed by flow cytometry exactly at 3 min after adding the acridine orange solution. Samples were run through Cytomics FC500 (Beckman Coulter, Inc.). Green fluorescence was detected using the FL-1 photodetector (530/28BP filter) and red fluorescence with the FL-3 photodetector (620SP filter). Data were collected from 10,000 events at 200 events/s for further analysis with Cell-Quest software (Becton Dickinson). At the beginning of each session, a standard semen sample was run through the cytometer, and settings were adjusted in order that mean fluorescence values (0-1023 linear scale) for FL-1 and FL-3 were 475 and 125, respectively. We calculated the DNA Fragmentation Index (DFI) of each event (spermatozoa) as the relation between red and total fluorescence, by means of the formula red / (red + green) × 100. Then, we classified the spermatozoa into three groups, according to DFI: normal DFI (<20% DFI), moderate DFI (from 20% to 75% DFI) and high DFI (from 75% to 100% DFI); we calculated the percentage of spermatozoa with a moderate (DFIm) and high (DFIh) DNA fragmentation index for

each sample. Total DNA fragmentation index (DFIt) was defined as DFIm + DFIh. We also calculated the mean of DFI (X-DFI) and standard deviation of DFI (SD-DFI) for each sample. In addition, we detected spermatozoa with high DNA stainability (HDS; FL1 channels above main population in FL1/FL3 flow cytometry dot plot). HDS spermatozoa corresponds to another distinct population in semen that characterizes immature spermatozoa (Fig. 2).

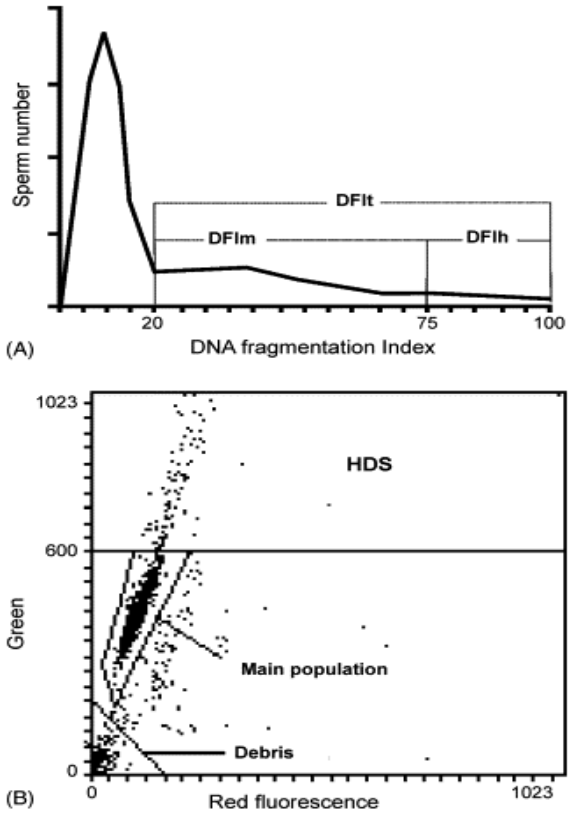


Fig. 2. Representation of DNA damage (SCSA)

(A) Schematic DFI histogram (red/red+ green fluorescence). Cells out of the main population are included in DFIt (fragmented DNA). (B) Cytographs of native DNA stainability (green fluorescence; FL1) vs. fragmented DNA (red fluorescence; FL3). Cells with green fluorescence above 600 belong to HDS (immaturity DNA).

(B) Cytogram representative of sperm chromatin structure assay (SCSA). Green fluorescence in ordinate represent double-stranded DNA and red fluorescence in abscissa represent single-stranded DNA showing DNA fragmentation index (%DFI) and high DNA stainability (%HDS).

2.6. Lead analysis.

Freeze-dried samples (0.5 g) of testicular parenchyma and epididymal cauda tissue were digested with 3 ml of HNO_3 (Suprapur 70%, Merk), 1 ml of H_2O_2 (30% v/v Suprapur, Merck) and 4 ml of deionized water with a microwave oven (Ethos E, Milestone). The program for the digestion began at a potency of 750 W and ramped for 15 min up to 180 °C, after which, samples were held for 10 min at 800 W and at a temperature of 180 °C. Digested tissue samples were diluted to a final volume of 15 ml with deionised water. Sperm suspensions (450 µl) were digested with 200 µl of deionized water and 200 µl of HNO_3 and 200 µl of H_2O_2 and left at room temperature for 24 h. Digested sperm suspensions were diluted to a final volume of 2 ml with deionized water. Blanks were processed with each batch of sample digestion.

Finally, the analysis of Pb was achieved using a graphite furnace-atomic absorption spectroscopy system (AAnalyst 800, Perkin Elmer) equipped with an autosampler (AS 800, Perkin Elmer) and using 50 µg of $NH_4H_2PO_4$ and 3 µg of $Mg(NO_3)_2$ as matrix modifiers in each atomization for Pb. Solutions used for calibration were prepared from commercial stock standards with 1 g/l of Pb (Panreac) and the limit of detection (LODs) were 12 ng/g dry weight (d.w.) in testicular parenchyma, 8 ng/g d.w. in epididymal caudae and 2 pg/10^6 spermatozoa in sperm suspension. A reference sample of bovine liver (BCR 185R, Community Bureau of Reference) with a certified Pb level of 172 ng/g d.w. was analyzed (n=8) and the recovery was 94.4±5.8%. Pb concentrations are given as ng/g d.w. of tissues and pg/10^6 spermatozoa in the sperm suspensions.

2.7. Statistical analysis.

Pb levels were log-transformed to attain or approach a normal distribution of the data. Values below the detection limits were assigned values of half of the respective LOD for statistical purposes. Differences in the studied parameter between control and mining areas were studied with Student t-tests. Moreover, the effects of the hunting season or the dates of sample collection in each season were studied with general linear models (GLM). Linear relationships correlations among Pb concentrations, chromatin damage indices, antioxidants and sperm quality parameters were studied with the Pearson's correlation coefficient. The significant relationships were then confirmed by means GLMs including the potential effects on sperm parameters (antioxidants, chromatin damage or sperm quality) as dependent variables and the predictors as covariants (Pb levels, antioxidants or chromatin damage) or factors (area). The criterion for significance was set at $p \leq 0.05$, but marginally significant effects ($p < 0.1$) have been also commented. The statistical analysis was performed with IBM SPSS Statistics v. 19.0.0.

3. Results.

Deer from the mining area showed higher Pb concentrations (log-transformed data) in testis parenchyma ($t_{56}=2.3$, $p=0.025$), epididymis ($t_{52.7}=4.2$, $p<0.001$) and spermatozoa ($t_{35.6}=4.9$, $p<0.001$) than in the control sites (Table 1). All the parameters of sperm quality were affected by mining pollution (Table 2). Red deer from the mining area showed lower quality of sperm movement ($t_{61}=2.0$, $p=0.05$) and lower percentages of sperm motility ($t_{61}=2.7$, $p=0.010$), viability ($t_{61}=2.3$, $p=0.027$) and acrosome integrity

(t_{61}=3.8, p<0.001) than in the control areas. In terms of oxidative stress biomarkers, X-DFI was higher in the spermatozoa of deer from the mining sites than in the controls (t_{61}=4.08, p<0.001; Table 3). HDS was also higher in the sperm samples from the mining area than in the controls (t_{61}=2.88, p<0.005; Table 3). Deer from the mining area showed higher GPX activity than in the control area (t_{61}=2.7, p=0.008), but no differences were observed for SOD activity or tGSH levels (Table 3). When season and date of sample collection were considered in the analysis with GLMs, only the differences in sperm viability and Pb in testis parenchyma became non-significant between areas. Differences in age groups (2-3 and 4-5 years) were non-significant for any of the studied parameters.

Table 1. Lead levels in testis tissues (ng/g d.w.) and spermatozoa (pg/10^6 spermatozoa) of red deer from control and mining areas.

Parameter	Control			Mines		
	N	Mean *GMean*	SE (min-max)	N	Mean *GMean*	SE (min-max)
Testis-Parenchyma	23	61 *58*	4 (27-105)	35	81 *73**	7 (24-206)
Testis-Epididymis	23	28 *27*	2 (19-47)	35	41 *39***	3 (16-99)
Sperm	26	10 *3*	2 (<2-39)	36	54 *30***	9 (2-153)

Significant differences in geometric means: *p≤0.05, **p≤0.001

Table 2. Parameters of sperm quality in red deer from control and mining areas.

Parameter	Control			Mines		
	N	Mean	SE	N	Mean	SE
Motility (%)	26	52.1	6.1	37	30.4**	5.3
Quality of movement (1-5)	26	2.3	0.3	37	1.6*	0.2
Acrosome integrity (%)	26	53.8	2.2	37	42.7***	1.9
Viability (%)	26	65.7	2.6	37	57.5*	2.4

Significant differences in arithmetic means: *p≤0.05, **p≤0.01, ***p≤0.001

Table 3. Parameters of DNA damage and antioxidants in spermatozoa of red deer from control and mining areas.

Parameter	Control			Mines		
	N	Mean	SE	N	Mean	SE
X-DFI (Mean)	26	23.2	0.2	37	24.5***	0.2
SD-DFI (SD)	26	2.45	0.12	37	2.61	0.13
DFIm (%)	26	4.33	0.58	37	4.60	.53
DFIh (%)	26	0.003	0.002	37	0.010	0.004
DFIt (%)	26	4.33	0.58	37	4.61	0.53
HDS (%)	26	3.21	0.34	37	4.63**	0.33
GPx (mU/10^6 spermatozoa)	26	0.963	0.196	36	1.605**	0.140
SOD (IU/ 10^6 spermatozoa)	26	0.063	0.008	37	0.072	0.010
tGSH (nmol/10^6 spermatozoa)	26	0.134	0.047	37	0.080	0.006

Significant differences in arithmetic means: *p≤0.05, **p≤0.01, ***p≤0.001

All the parameters of sperm quality were correlated among them (all r>0.456 and p<0.001). GPx activity in spermatozoa was negatively correlated with sperm motility (r=-0.521, p<0.001), quality of movement (r=-0.514, p<0.001), acrosome integrity (r=-0.277, p=0.029; related to area in GLM) and viability (r=-0.614, p<0.001).

X-DFI was also negatively correlated with sperm motility (r=-0.366, p=0.003), quality of movement (r=-0.295, p=0.019), acrosome integrity (r=-0.533, p<0.001; see Fig. 3a for GLM analysis) and viability (r=-0.334, p=0.008). X-DFI was not associated with GPx activity, but it was positively correlated with SOD activity in spermatozoa (r=0.338, p=0.007) and negatively with tGSH levels (r=-0.251, p=0.047; related to area in GLM). Moreover, SOD activity was positively correlated with GPx activity in spermatozoa (r=0.275, p=0.031). HDS was positively correlated with X-DFI (r=0.560, p<0.001), negatively with motility, quality of movement and acrosome integrity (all with r<-0.3 and p≤0.017), and positively with GPx and SOD activities (both r>0.25 and p<0.05). SD-DFI was negatively correlated with sperm motility, quality of movement, acrosome integrity (all with r<-0.31 and p≤0.014), and positively with GPx activity (r=0.369, p=0.003). Lead concentration in sperm was negatively correlated with all the parameters of sperm quality (all with r<-0.41 and p≤0.001; see Fig. 3b for the GLM with acrosome integrity) and positively with GPx activity (r=0.658, p<0.001), X-DFI (r=0.384, p=0.002; but this was mostly related to area as shown in GLM analysis, see Fig 3c) and SD-DFI (r=0.355, p=0.005). Lead levels in testis parenchyma were negatively correlated with acrosome integrity (r=-0.271, p<0.039) and tGSH levels (r=-0.296, p<0.024). Finally, Pb levels in testis parenchyma and epididymis were positively correlated (r=0.588, p=0.002), but these two were only marginally correlated with Pb levels in spermatozoa (both with p<0.1).

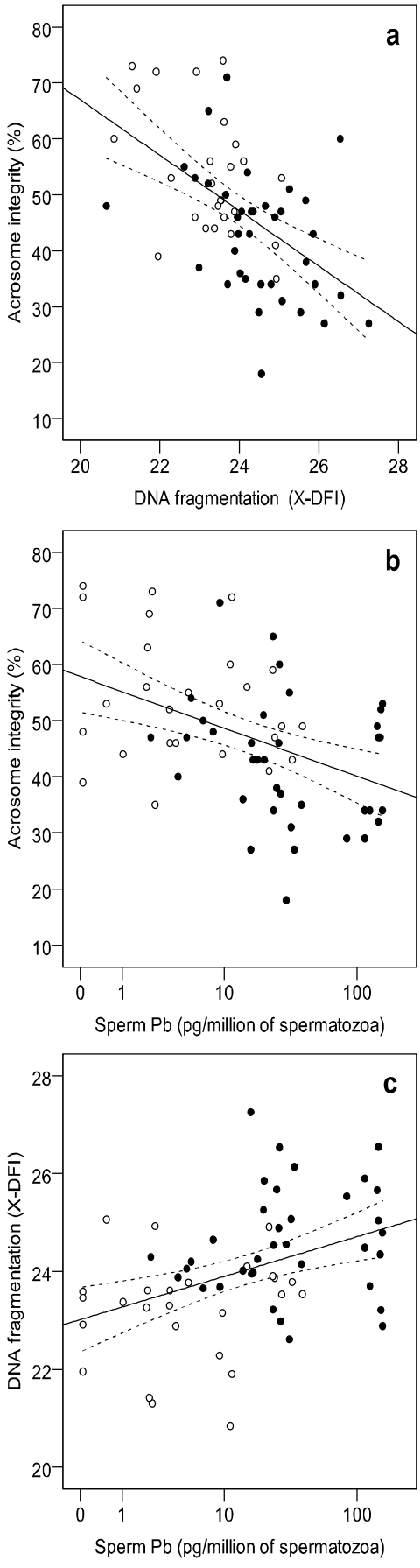


Fig.3. Relationships between Pb levels, acrosome integrity, and DNA fragmentation (X-DFI) in spermatozoa of red deer from Pb polluted (closed circles) and control (open circles) sites. GLMs have shown significant relationships between these three parameters and significant effects of the area. Fig. 3a: Acrosome integrity was negatively related with X-DFI (Wald's χ^2_1=12.96, p<0.001) and the mining activity (area) (Wald's χ^2_1=12.96, p<0.001). Fig. 3b: Acrosome integrity was negatively related to sperm Pb levels (Wald's χ^2_1=3.13, p=0.077) and significantly affected by mining activity (area) when both variables were included together in the model (Wald's χ^2_1=5.12, p=0.023). Note that Sperm Pb was also associated by mining activity. Fig. 3c: DNA fragmentation (X-DFI) was positively correlated with sperm Pb levels (r=0.384, p=0.002), but GLM showed that this effect was related to the area (Wald's χ^2_1=12.23, p<0.001) and not to sperm Pb levels (Wald's χ^2_1=0.89, p=0.345). This may indicate that other metals and metalloids not measured here could also contribute on the observed chromatin damage.

4. Discussion.

The contamination caused by old mining activities in Alcudia Valley – Sierra Madrona is reflected nowadays in high levels of Pb and other metals in red deer tissues, including different parts of testis and spermatozoa. As observed in previous studies (Reglero et al., 2009b), deer from this mining area showed lower values of acrosome integrity. This effect was here accompanied by higher values of DNA fragmentation (X-DFI) and stainability (HDS). Interestingly, these three parameters were significantly correlated among them showing the potential adverse effect of Pb in spermatozoa by producing chromatin damage and a loss of the acrosome integrity. Moreover, deer from the mining area showed higher GPx activity, which could be because of the effort to cope with the oxidative damage induced by Pb.

Red deer from the mining area of Alcudia Valley – Sierra Madrona have higher levels of Pb in liver, spleen and bone than deer from control areas (Reglero et al., 2008, 2009a; Rodríguez-Estival et al., 2011, 2013); but no differences had been described before in Pb levels in testis and sperm. Nevertheless, Pb is not the only metal present at elevated levels in deer from the study area. Higher hepatic levels of Cu, As and Se and, especially, Cd have been observed in deer from the mining area before (Reglero et al., 2008, 2009a; Rodríguez-Estival et al., 2011). This is remarkable, because the exposure to both Pb and Cd has been associated with disrupted initiation and duration of acrosome reaction in mammalian spermatozoa (Marzec-Wróblewska et al., 2012). Nevertheless, the accumulation of some elements observed in liver may not occur always in testis or spermatozoa, and this may have a relationship with the activity of antioxidant enzymes that require the presence of specific elements (i.e. GPx or SOD). In the case of Cu, the levels in the testis of deer from the same Pb polluted area were lower

than in control areas, and this negative effect on Cu homeostasis was significantly associated with lower SOD activity in testis and sperm viability (Reglero et al., 2009b). In the present study, SOD activity in the spermatozoa was not affected by the mining pollution. The lower GPx activity in testis in red deer from the mining area observed by Reglero et al. (2009b) contrasts with the higher GPx activity observed here in the spermatozoa. These differences in the effects of mining pollution between testis parenchyma and sperm may respond to an attempt to protect spermatozoa over parenchymal testis with this antioxidant enzyme. In this sense, ultraestructural damage in testicular cells has been observed in rats exposed to Pb, but without significant changes in sperm motility and count (Murthy et al., 1995).

Several studies have related adverse effects of heavy metals on sperm quality with oxidative stress. Bovine sperm with 0.23-0.57 µg/ml of Pb have shown a reduced fertilization capacity and an altered prooxidant-antioxidant balance, as evidenced the decrease in GSH and total antioxidant status (TAS), and the increase in lipid peroxidation measured as malondialdehyde (MDA) levels (Tvrda et al., 2012, 2013). Humans with elevated levels of Cd and Pb in sperm have also shown reduced fertility accompanied by higher MDA, protein carbonyls and ROS levels, and lower GSH levels and GSH-*s*-transferase (GST) activities, suggesting an oxidative damage of these heavy metals on lipids and proteins (Kiziler et al., 2007). Spermatozoa of red deer, as well as in other animal species, contain high levels of polyunsaturated fatty acids (PUFAs) in their membrane (Castellanos et al., 2010), which makes them more susceptible to the membrane lipid peroxidation (Wathes et al., 2007). Red deer from the mining area studied here have shown a decrease in testis and spermatozoa of the percentage of arachidonic acid (20:4n-6) (Castellanos et al., 2010), which is the precursor of eicosanoids involved in the acrosome reaction (Breitbart and Spungin, 1997).

Apart of lipids and proteins, DNA is another of the target molecules of oxidative stress. Although ROS are required for critical aspects of sperm function, excessive levels can induce oxidation of critical sulfhydryl groups (-SH) in proteins and DNA and increase the formation of 8-hydroxy-deoxyguanosine (8-OhdG), which can alter the integrity and function of spermatozoa (Kefer et al., 2009). Increased DNA strand breaks and poor DNA packing quality have been associated with elevated ROS production and poor sperm quality in humans and animals (Filatov et al., 1999; Irvine et al., 2000; Moustafa et al., 2004; Nagy et al., 2013). Environmental pollutants, including Pb and other heavy metals, are known to cause sperm DNA fragmentation and infertility (Oliva et al., 2001; Taha et al., 2013). *In vitro* studies have shown the alteration of the DNA-protamine binding by Pb interaction with -SH of these proteins, that may result then in chromatin alterations and DNA damage (Quintanilla-Vega et al., 2000). Moreover, Pb has genotoxic effects by its accumulation in the cell nucleus and by inducing non-genotoxic/epigenetic mechanisms that affect DNA and even its synthesis (Silbergeld et al., 2000) or by interfering DNA repair and therefore increasing the susceptibility to other genotoxic agents (Hartwig, 1994). As we have observed in the red deer of our study, the animals can be exposed to several toxicants that can exert adverse effects by synergistic or additive mechanisms (Carpenter et al., 2002). Therefore, there can be interactive effects of toxicants, such as Cd and Pb can do in the mining area studied here, leading to the induction of DNA damage as observed in humans with occupational exposure to Pb and Cd (Palus et al., 2003).

The analysis of the degree of DNA fragmentation by SCSA is considered to be the most precise and repeatable assay, with better predictive value for the detection of male infertility than classical semen analyses (Evenson, 2013). This technique has been also employed to asses toxicant-induced sperm chromatin damage (Evenson, 2013) and

allows to detect epigenetic changes and meiotic alterations resulting in elevated proportions of diploid spermatozoa and infertility (Nagy et al., 2013). We have observed 5.6% higher X-DFI and 44% higher HDS values in the deer from the mining area than in the control area. Function of spermatozoa with X-DFI values above 30% can be greatly compromised (Bungum et al., 2007), but deer from the mining (24.5%) and control (23.2%) areas showed values below this threshold. The X-DFI was found to be the SCSA parameter for assessing sperm chromatin damage that was better associated to mining pollution in red deer of our study. This parameter is known to increase significantly in bull spermatozoa exposed to an oxidant treatment with Fe^{2+} (Martínez-Pastor et al., 2009). In men with clinical varicocele, the elevated presence of ROS in normal and abnormal ejaculated sperm has been accompanied with high DNA DFI and apoptosis (Smith et al., 2006). On the other hand, testis parenchyma has very high rates of cell proliferation and this requires of the elimination of excess germ cells by means a natural apoptosis in the testis by cutting of DNA into discrete size in early germ cells to ensure an efficient spermatogenesis. However, a few defective sperm cells escape this elimination and produce immature forms of spermatozoa with high levels of DNA fragmentation (Aziz et al., 2007). Hence, the presence of fragmented DNA in sperm due to residual apoptotic cells passing into the seminiferous tubes adds to those produced by genotoxic agents (Sjöblom et al., 1998). The higher HDS in the spermatozoa in red deer of the mining area observed here can be interpreted as a higher presence of immature spermatozoa in the epididymis than in the control sites (Elshal et al., 2009).

5. Conclusions.

The adverse effects of mining pollution on higher vertebrates are usually sublethal. We have detected here by means of chromatin damage biomarkers that DNA alterations may exist in spermatozoa even at these sublethal levels of exposure with potential consequences on the reproduction and the offspring. The observed damage in chromatin condensation could have been triggered by oxidative stress, because deer from the mining area showed a higher GPx activity that may reflect an attempt to maintain the redox balance in spermatozoa. The study also confirms that the loss of acrosome integrity is a common finding in deer from an old mined site where Pb is the most important pollutant, but not the unique, remaining in the environment.

Acknowledgements

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CAPÍTULO 4

Identification of Optimal Concentrations and Incubation Times for the Study of *In vitro* Effects of Pb in Ram Spermatozoa

Morueco en rebaño de oveja manchega. Foto: Vicente Luchena

Castellanos P, Maroto-Morales A, García-Álvarez O, Garde JJ, Mateo R. 2013. **Identification of Optimal Concentrations and Incubation Times for the Study of In Vitro Effects of Pb in Ram Spermatozoa.** *Bulletin of Environmental Contamination and Toxicology* 91(2): 197-201.

Identificación de la concentración y tiempo de incubación óptimos para el estudio *in vitro* de los efectos del plomo en el espermatozoide de morueco (*Ovis aries*).

RESUMEN

Se han estudiado los efectos del plomo (Pb) *in Vitro* sobre los espermatozoides de morueco (*Ovis* aries) con la finalidad de establecer un nivel umbral que afecte a la función espermática. Para ello se han incubado los espermatozoides durante diferentes tiempos entre 15 y 180 minutos y con distintas concentraciones de plomo en un rango entre 0 y 5000 ng/ml Pb2+. Tanto la presencia de plomo como el tiempo de incubación han afectado negativamente a la motilidad, la integridad del acrosoma, la funcionalidad de la membrana y la viabilidad del esperamtozoide.

La integridad del acrosoma ha estado linealmente afectada por ambos factores siendo a los 30 minutos de incubación y con 50 ng/ml de Pb2+, el tiempo y concentración de Pb más bajos a los que se ha producido este efecto.

De esta forma, se ha visto que estos valores de concentración y tiempo de incubación con plomo pueden representar unas condiciones experimentales apropiadas para los estudios *in Vitro* de los mecanismos de acción del plomo sobre los espermatozoides.

***Palabras Clave*:** plomo, espermatozoide, calidad seminal, integridad del acrosoma.

ABSTRACT

In vitro effects of lead (Pb) on ram (Ovis aries) spermatozoa were studied to establish a threshold level that affects sperm function. Spermatozoa were incubated between 15-180 min with Pb concentrations ranging from 0 to 5,000 ng/ml. Sperm motility, acrosome integrity, membrane functionality and sperm viability were all negatively affected by Pb and incubation time. Acrosome integrity was linearly affected by Pb levels at an incubation time of 30 min, and 50ng/ml de Pb^{2+} was the lowest Pb level producing such effect. These experimental conditions can be appropriate for in vitro studies of the mechanisms of action of Pb on spermatozoa**.**

Key words**:** lead, spermatozoa, semen quality, acrosome integrity.

1. Introduction.

Several studies have suggested that the semen quality in humans is globally declining due to different environmental factors such as lifestyle, nutrition or exposure to chemical substances (Skakkebaek et al. 2006; Deonandan and Jaleal 2012). The potential deleterious role of toxic compounds in this progressive alteration of men reproduction function has been supported by experimental studies with animals and similar evidences observed in wildlife (Auger 1997). Particularly, the increase of heavy metal emissions in developed countries and the occupational exposure to lead (Pb) have been associated with adverse effects on male reproductive function. Pant et al. (2003) showed a significant negative association between Pb concentrations in semen and sperm quality in humans, having found 60 ng/ml of Pb in seminal plasma of fertile men and 125 ng/ml in infertile oligospermic, asthenospermic and azoospermic men. Xu et al. (2003) have associated a Pb concentration in human seminal plasma of 10 ng/ml with lower sperm density and higher oxidative DNA damage in spermatozoa. Lead neurotoxicity can also negatively affect the male reproductive function through an imbalance of the hypothalamic-pituitary-gonad axis. Rats exposed to lead acetate in drinking water (0.3-0.6%) during their development that had a mean blood Pb of 300 ng/ml showed a significant decrease in serum and intratesticular testosterone levels and a decrease in sperm count by a suppression of spermatogenesis (Ronis et al. 1996). However, few studies have been focused on the biochemical effects of Pb after spermatogenesis, and particularly on membrane properties associated with sperm quality (Benoff et al. 2003a). In a previous study we observed an immediate reduction in the acrosome integrity of ram spermatozoa exposed *in vitro* to 50-500 ng/ml of Pb^{2+} (Castellanos et al. 2008). In the present study, we have assessed *in vitro* effects of Pb in

ram spermatozoa to determine a threshold Pb level appropriate to study the mechanism of action of Pb on spermatozoa when it contacts with seminal plasma or fluids of the female genital tract.

2. Materials and methods.

Semen was collected by artificial vagina from six healthy rams (*Ovis aries*) of Manchega breed from the Animal Reproduction Centre (CERSYRA, Valdepeñas). Sperm is routinely collected in the Manchega males of this Centre for the artificial insemination of the herds of Castilla-La Mancha region. Therefore, these males have been previously evaluated and selected by their quality as donors. We collected two or three ejaculates per animal and the one with the largest volume and best wave motion (above 3) was used for the experiment. The collected volume, sperm concentration, wave motion, individual sperm motility and quality of movement were evaluated. Sperm concentration was determined by transmittance with a spectrophotometer (Type Helios Delta model, Thermo Electron Corporation), diluting 5 µl of ejaculates in 5 ml of sodium citrate 128 mM. Wave motion was subjectively scored from 1 to 5, where 0 is no movement and 5 is strong wave movement, on a wet mount of neat semen using bright field microscopy at ×10 (BH-2 Olympus). Individual sperm motility and quality of movement were also assessed, 5 µl of semen were diluted on 200 µl of phosphate-buffer saline (PBS). After incubation at 37 ºC for 5 minutes, 5 µl of sperm diluted were placed between a pre-warmed slide and a 22 mm × 22 mm coverslip and were observed at ×400 under phase-contrast optics (Eclipse 50i Nikon). The percentage of motile sperm was estimated subjectively by three experienced observers after any drifting of the specimen due the placement of the coverslip had stopped. The percentage of motile

spermatozoa was determined to the nearest 5% by analysing four to five fields of view (Evans and Maxwell 1987) under the microscope with values ranging from 0 %, when no motile spermatozoa were observed, to 100 %, when all spermatozoa were moving. Quality of sperm movement was also estimated subjectively on a scale of 0 – 5, where 0 is no motility and 5 is vigorous progressive movement.

Samples of each ram were diluted in six aliquots of 5 ml to obtain 75×10^6 spermatozoa/ml with the modified tyrode medium supplemented with 10 mM sodium pyruvate (Sigma Ultra 99%) and 6 mg/ml of bovine serum albumin (BSA) (albumin bovine serum, factor V 96%, Sigma). Pb acetate, (lead II acetate tri-hydrate 99%, Aldrich) was previously added to the incubation media to obtain six treatments per ram with spiked Pb^{2+} concentrations of 0, 0,5, 5, 50, 500 or 5,000 ng/ml of Pb^{2+} (0, 0.0024, 0.024, 0.24,2.4 or 24 µM). Then, each of the six tubes of each ram was divided in five aliquots of 1 ml for incubation during 15, 30, 60, 120 or 180 minutes at 37.5 ºC.

Sperm quality parameters were measured in 30 µl of each aliquot at the corresponding incubation time at 37 ºC. Individual sperm motility and quality of movement was evaluated as above mentioned. Plasma membrane functionality was assessed by means of the hypo-osmotic swelling test as described by Garde et al. (1998). Briefly, 10 µl of diluted sperm sample was mixed with 0.1 ml of hypo-osmotic solution (sodium citrate, 100 mOsmol/kg) and incubating the mixture at room temperature (≈23 ºC) for 30 min. The samples were then fixed in 2% glutaraldehyde in 0.165 M cacodylate/HCl buffer (pH 7.3) and evaluated at ×400 under phase-contrast optics. The sperm membrane was considered intact if the sperm tail was coiled at the end of the assay and the result was expressed as % positive endosmosis. Sperm viability was evaluated by means of a nigrosin–eosin stain (NE). The NE stain was prepared as described by Tamuli and Watson (1994). The diluted sperm (5 µl) was mixed with the

NE stain (10 µl) at 37 °C, incubated for 30 sec, smeared and dried on a warm plate at 37 °C. The samples were evaluated using bright field microscopy at ×400. Live spermatozoa remained unstained, while dead cells were dull pink. The % of live spermatozoa was expressed as viability. Acrosomal integrity was evaluated after a 1:10 dilution in 2% glutaraldehyde/cacodylate, as above. The % of spermatozoa with intact acrosomes (i.e., with normal apical ridges) was assessed at ×400 under phase-contrast optics. Membrane functionality, viability and acrosomal integrity were studied in 100 spermatozoa per sample.

The remaining 0.97 ml of six lead treatment samples of each animal incubated during 180 min were centrifuged at 940 rcf for 10 min, the supernatant removed and the remaining pellet washed with 1 ml of cold saline solution. After a second centrifugation the pellet was kept frozen at -80 ºC for Pb analysis. Before Pb analysis, the pellet was mixed with 200 µl of deionized water and transferred to quartz tubes. Then, 2 ml of HNO_3 (nitric acid suprapur 70%) were added and left at room temperature during 12 hours. After this, 2 ml of H_2O_2 (30% hydrogen peroxide) were added and the tubes were gradually heated during 1 h in a standard heatblock (VWR) up to 150 °C then this temperature was maintained during 3 h. Finally, the digested sample was brought to a final volume of 5 ml with deionized water. Pb analysis was performed by graphite furnace atomic absorption spectroscopy (AAnalyst 800, Perkin Elmer) using 50 µg of $NH_4H_2PO_4$ and 3 µg of Mg $(NO_3)_2$ as matrix modifiers in each atomization. Solutions used for calibration were prepared from a commercial standard with 1 g/l of Pb. The limit of detection calculated as three times the standard deviation of blanks of digestion was 0.59 ng/ml. A reference material of blood (Community Bureau of Reference-BCR 190 with certified value of 772 ng/ml) was also analyzed and the recovery obtained for Pb was 104.3%.

The effects of Pb concentrations on sperm motility, viability and acrosomal integrity were studied with General Linear Models (GLM), including Pb concentration, incubation time and individual as factors and including their first-order interactions. Least Significant Difference (LSD) test was used to establish post-hoc differences between the values found at the different Pb concentrations and controls. Sperm Pb levels at the end of the incubation experiment were log-transformed to attain a normal distribution and these values were compared between Pb-exposure levels with a One-way Analysis of Variance (ANOVA) with a post-hoc LSD test. The level of significance was set at $p < 0.05$. These analyses were performed with IBM SPSS Statistics v. 19.

3. Results and Discussion.

The initial values of sperm parameters from rams #1 to #6 before incubation with Pb were as follows; wave motion: 4, 3.5, 3, 3, 3.5 and 3; individual motility: 80, 75, 75, 75, 50 and 50%; quality of movement: 4, 3.5, 3, 3.5, 2 and 2.5; and spermatozoa concentrations were 4,946, 3,961, 3,814, 4,304, 4,030, 2,887 $\times 10^6$ spermatozoa/ml, respectively.

Several parameters of sperm quality have been negatively affected by the incubation time and the presence of Pb in the medium, but the interactions of these two factors were not significant (Table 1, Fig. 1). Moreover, there was a significant effect of the individual on the sperm quality parameters, and the effect of incubation time on sperm quality parameters differed among individuals as the interaction between these two factors reflects (p values between <0.001 and 0.034). On the contrary, the

interaction between the individual and Pb was not significant in any of the studied parameters.

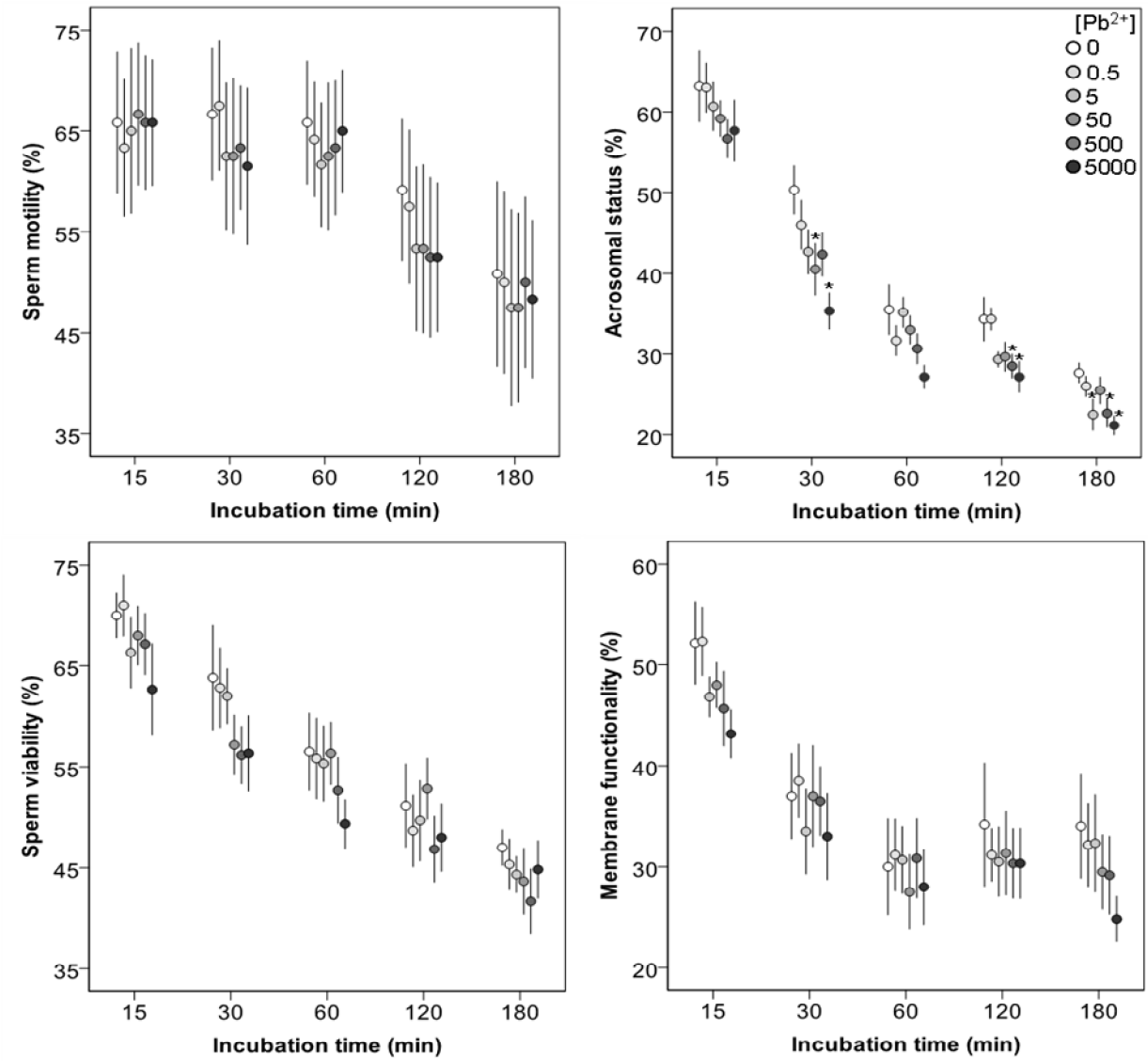

Fig. 1. Effect of Pb concentration and incubation time upon different spermatozoa parameters (mean±SE of six rams). Acrosomal status was affected by Pb concentrations at incubation times of 30 min ($F_{5,30}$=3.173, p=0.020), 120 min ($F_{5,30}$=2.88, p=0.031) and 180 min ($F_{5,30}$=2.661, p=0.042). Asterisks denote differences respect to the control at the corresponding incubation time.

Table 1. Results of the general linear models used to test the effects of the lead exposure level, incubation time, individual, and their first order interactions (independent variables) on the sperm quality parameters (dependent variables) of ram spermatozoa treated with lead acetate.

Independent variables	Dependent variables				
	Motility	Quality of motility	Sperm viability	Membrane functionality	Acrosomal status
Time	$F_{4,20}$=20.85 p<0.001	$F_{4,20}$=20.6 p<0.001	$F_{4,20}$=84.26 p<0.001	$F_{4,20}$=20.97 p<0.001	$F_{4,20}$=80.28 p<0.001
Pb	$F_{5,25}$=3.21 p=0.023	NS	$F_{5,25}$=4.77 p=0.003	$F_{5,25}$=4.63 p=0.004	$F_{5,25}$=12.85 p<0.001
Ram #	$F_{5,22}$=98.26 p<0.001	$F_{5,20}$=71.3 p<0.001	$F_{5,20}$=30.89 p<0.001	$F_{5,20}$=16.23 p<0.001	NS
Time × Pb	*ns*	*ns*	*ns*	*ns*	*ns*
Time × Ram #	$F_{20,100}$=7.26 p<0.001	$F_{20,100}$=5.92 p<0.001	$F_{20,100}$=1.77 p=0.034	$F_{20,100}$=4.32 p<0.001	$F_{20,100}$=5.02 p<0.001
Pb × Ram #	*ns*	*ns*	*ns*	*ns*	*ns*

ns = not significantly different by area using a nested generalized linear model (GLM).

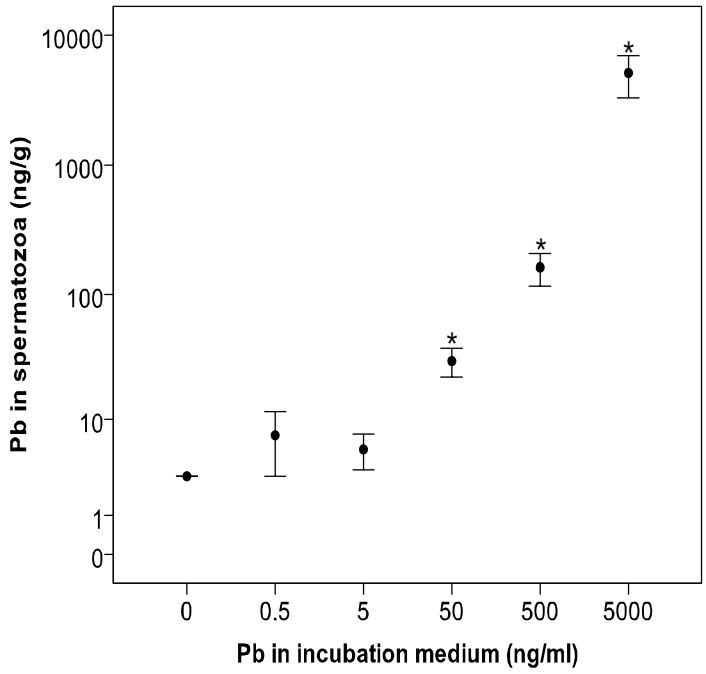

Fig. 2. Lead concentration in spermatozoa (mean±SE of six rams) after incubation with different Pb concentrations ($F_{5,30}$=72.74, p<0.001). Asterisks denote differences respect to the control.

Sperm motility was significantly reduced by increasing Pb concentration in the incubation medium ($p=0.023$), with incubation time ($p<0.001$) and by individual ($p< 0.001$). The quality of motility was also affected by incubation time ($p<0.001$) and individual ($p<0.001$), but not by Pb concentration. Viability was markedly reduced by incubation time ($p<0.001$), individual ($p<0.001$) and Pb concentration ($p=0.003$). Similarly, membrane functionality was affected by incubation time ($p<0.001$), individual ($p<0.001$) and Pb concentration ($p=0.004$). Acrosomal integrity was also affected by incubation time ($p<0.001$) and Pb concentration ($p<0.001$), but not by individual ($p=0.081$) (Table 1). Acrosomal integrity was the parameter that showed a better dose-response in relation to Pb concentration in the incubation medium, especially at 30 min of incubation (Fig. 1). Acrosomal integrity was significantly lower than in the control at Pb concentrations of 50 and 5,000 ng/ml. This parameter was also significantly reduced respect to the control at 120 min of incubation with 500 and 5,000 ng/ml of Pb and at 180 min with 5, 500 and 5,000 ng/ml of Pb (Fig. 1).

The presence of Pb in the incubation medium was associated with elevated Pb levels in spermatozoa after incubation during 180 minutes, especially at Pb concentrations in the medium ≥50 ng/ml ($p<0.001$; Fig. 2). Pb present in the spermatozoa pellet was (mean±SE) 6.3±1.3% of the total Pb added to the incubation medium with ≥50 ng/ml. The mass of the spermatozoa pellet was 103±13 mg. In terms of ng Pb/10^6 spermatozoa, the detected values for samples incubated with 0, 0.5, 5, 50, 500 and 5,000 ng/ml were <0.002, 0.008±0.005, 0.005±0.001, 0.026±0.010, 0.129±0.051 and 3.157±0.768, respectively.

Although most of the experimental work in rodents have associated blood Pb levels of >300-400 ng/ml with impairment of spermatogenesis (Apostoli et al. 1998), blood Pb levels as low as 49 ng/ml have been related in non-occupationally exposed

men with an increased presence in semen of immature sperm cells and with abnormal morphology, a decrease of Zn in seminal plasma, an increase of serum levels of testosterone and estradiol, and a decrease in serum level of prolactin (Telisman et al. 2007). Lead levels in sperm and seminal plasma are markedly lower than in blood. Hernández-Ochoa et al. (2005) have found in urban men a geometric mean of Pb levels in blood of 93.1 ng/ml that corresponded to 0.047 ng/10^6 spermatozoa and 2.02 ng/ml of seminal fluid. In fact, seminal plasma and spermatozoa Pb levels may be better determinants of sperm quality than blood Pb levels. The minimum concentrations of Pb in human spermatozoa associated with abnormal semen quality were 0.07 ng /10^6 spermatozoa for a negative effect on sperm motility and 0.24 ng /10^6 spermatozoa for reduced sperm viability (Hernández-Ochoa et al. 2005). In our experiment, final Pb concentrations in spermatozoa incubated in medium with 50 ng/ml of Pb was 0.026 ng /10^6 cells, which is slightly lower than the level observed *in vivo*. Nevertheless, the concentration of Pb in the incubation medium was much higher than the values detected in seminal plasma, probably because Pb uptake in spermatozoa was more effective *in vivo* than in our *in vitro* model.

Other *in vitro* studies have evaluated the effects of Pb on spermatozoa. Rabbit spermatozoa incubated in a medium with 5,170 ng/ml of Pb showed a decrease in the fertilization rate (Foote, 1999). Bull spermatozoa incubated with 2,500 ng/ml of Pb showed a decrease in the sperm motility but no effect was observed at 250 ng/ml (Alexaki et al. 1990). Buffalo spermatozoa incubated with 100 ng/ml for 1 h showed a reduced acrosomal integrity (Selvaraju et al. 2011). Here, we have observed an adverse effect on acrosomal integrity at lower Pb levels than those reported in other studies. On the other hand, Benoff et al. (2003b) exposed human sperm to Pb concentrations similar

than in our experiment (51.5-5,180 ng/ml) and observed an increase in the spontaneous acrosome reaction in a dose-dependent manner.

A premature acrosome reaction or a reduced acrosomal integrity have also been reported in spermatozoa of Pb-exposed males of different species. In wild ungulates such as red deer, the percentage of acrosomal integrity in spermatozoa collected from the cauda epididymae was lower in Pb-polluted areas (79%) than in control areas (90%) (Reglero et al., 2009). These Pb-exposed deer also had lower Cu levels and higher Se levels in testis, decreased activities of SOD and GPX in testis and spermatozoa, and lower levels of arachidonic acid in testis and spermatozoa (Reglero et al. 2009; Castellanos et al. 2010). Sperm Pb level in deer from these Pb-polluted sites was 0.003 ng/10^6 cells (Reglero et al. 2009), which is lower than the resultant level in ram spermatozoa incubated here with 50 ng/ml. In the case of rodents, Hsu et al. (1998) found that the percentage of acrosome-reacted spermatozoa was significantly higher in rats receiving weekly an intraperitoneal injection of 50 mg/kg of Pb acetate for 6 weeks (7.5%) than in the controls (1.7%). Johansson (1989) also observed a premature acrosome reaction in spermatozoa of Pb-exposed mice and this reduced their ability to fertilize oocytes. In humans, Pb seminal plasma levels ranging from 100 to 1,500 ng/ml were negatively correlated with the artificial insemination rate and positively correlated with a premature acrosome reaction (Benoff et al. 2003a).

4. Conclusions.

In conclusion, *in vitro* Pb-exposure of spermatozoa in an incubation medium with 50 ng/ml of Pb during 30 min can be appropriate for the study of the mechanism of action of extracellular Pb on spermatozoa, especially for a target such as the acrosomal status and its impact on fertility.

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CAPÍTULO 5

In vitro effects of lead on fatty acid composition, oxidative stress biomarkers and quality of ram spermatozoa

Oveja manchega en cría extensiva. Foto: Vicente Luchena

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Efectos del plomo *in vitro* en la composición de ácidos grasos, biomarcadores de estrés oxidativo y calidad del espermatozoide de morueco.

RESUMEN

Se ha evaluado el efecto del plomo sobre la calidad espermática y sobre varios parámetros asociados con el estrés oxidativo en suspensiones de espermatozoides de morueco (*Ovis aries*) incubadas con 0, 50 o 500 ng/ml de Pb^{2+} durante 0, 1, 3 y 6 horas. La presencia de Pb en el medio de incubación ha reducido la integridad del acrosoma y la motilidad de los espermatozoides así como la funcionalidad y la integridad de su membrana. Por el contrario, el porcentaje de ácidos grasos poliinsaturados (PUFAs) y la actividad de las enzimas glutatión peroxidasa (GPx) y superóxido dismutasa (SOD) no se vieron afectadas por la presencia de plomo.

Sin embargo, la actividad de la GPx descendió y la composición total de ácidos grasos cambió al aumentar la proporción relativa de ácido esteárico (18:0) durante el tiempo de incubación pero de forma independiente a la presencia de plomo.

Los datos muestran que varios de los efectos del plomo sobre la calidad espermática que se observan normalmente *in vivo* también se han registrado en este estudio *in Vitro*, pero en este caso sin guardar relación con los biomarcadores de estrés oxidativo.

***Palabras clave*:** plomo, metales pesados, calidad espermática, estrés oxidativo, ácidos grasos.

ABSTRACT

The sperm quality and several parameters associated with oxidative stress were evaluated in ram (*Ovis aries*) spermatozoa suspensions incubated with 0, 50 or 500 ng/ml Pb during 0, 1, 3, or 6 hr. The presence of Pb during incubation reduced the integrity of the acrosome, % sperm motility and the integrity and the functionality of membrane. On the contrary, % polyunsaturated fatty acids and the activities of glutathione peroxidase and superoxide dismutase in the spermatozoa suspensions were not affected by Pb. Moreover, glutathione peroxidase activity decreased and fatty acid composition changed due to the relative increase in % stearic acid during the incubation time independently on Pb presence. Data showed that several effects of Pb on sperm quality usually observed in vivo also occurred in vitro, but without any relationship with oxidative stress biomarkers.

Keywords: lead, heavy metals, sperm quality, oxidative stress, fatty acids.

1. Introduction.

The male reproductive function is affected by lead (Pb) and other heavy metals (Rom, 1980; Benoff et al., 2000; Pant et al., 2003; Xu et al., 2003). Men with >400 ng/ml of Pb in blood due to occupational exposures have reduced sperm count, volume and density, or changing sperm motility and morphology (Lancrajan et al., 1975; Apostoli et al., 1998). Rodents with >300-400 ng/ml of Pb in blood have experimentally shown impairment of spermatogenesis, reduced concentrations of androgens and premature acrosome reaction (Hilderbrand et al., 1973; Sokol et al., 1985; Johansson, 1989; Hsu et al., 1998a; Apostoli et al., 1998). Several studies observed higher seminal Pb concentrations in infertile than in fertile men from Finland (3.6 vs. 1.7 ng/ml) (Saaranen et al., 1987), Egypt (196 vs. 111 ng/ml) (El-Zohairy et al., 1996) and India (60 vs. 125 ng/ml) (Pant et al., 2003). However, other studies found no significant differences between fertile and infertile men (Umeyama et al., 1986). In general, Pb levels in seminal plasma are taken into account to evaluate semen donors (Benoff et al., 2003b) or in infertility studies (Benoff et al., 2003a).

The effects of Pb on spermatozoa functions were studied *in vitro* with sperm of humans (Huang et al., 2001; Benoff et al., 2003a), bull (Alexaki et al., 1990) and rabbit (Foote, 1999). The reduction of sperm motility and the increased spontaneous acrosome reaction are some of the effects of Pb observed *in vivo* that were reproduced *in vitro*. These *in vitro* studies permitted one to identify some mechanisms of action of Pb on human spermatozoa. Benoff et al. (2003a) observed a decrease of mannose receptor expression and motility in human spermatozoa incubated with Pb in a concentration-dependent manner from 51.5 to 5180 ng/ml. Moreover, the incubation of human spermatozoa with 2.5 µM (517 ng/ml) of Pb induced a spontaneous loss of the acrosome

that was inhibited by the addition of charybdotoxin, an inhibitor of voltage-gated potassium channels (Benoff et al., 2000).

Pb exposure induces oxidative stress in several tissues and cells, including spermatozoa (Acharya et al., 2003; Mateo et al., 2003b). The spermatozoa are especially susceptible to oxidative stress due to the high presence of polyunsaturated fatty acids (PUFA) in their membranes (Giannattasio et al., 2002; Vernet et al., 2004). In human spermatozoa, 40% fatty acids are PUFA, which are responsible for membrane fluidity (Poulus et al., 1973). The generation of reactive oxygen species (ROS) in the sperm of Pb-exposed mice was associated with an increase of acrosome reacted spermatozoa (Hsu et al., 1998a), suggesting the implication of oxidative stress in the premature capacitation induced by Pb.

In the present study the changes in sperm characteristics were evaluated when ram spermatozoa were exposed *in vitro* to different concentrations of Pb and how these changes might be related to oxidative stress or to changes in the fatty acid composition of spermatozoa. The exposure *in vitro* of ejaculated spermatozoa may help to understand the direct effects of Pb after spermatogenesis.

2. Materials and methods.

All chemicals for sperm incubation and evaluation were of reagent grade and were purchased from Sigma or Merck (both of Madrid, Spain).

2.1. Sampling and incubation conditions

Animal manipulations were performed in accordance with the Spanish Animal Protection Regulation, RD223/1988, which conforms to European Union Regulation 86/609. Semen was collected by electroejaculation of two healthy rams (*Ovis aries*) and immediately evaluated for volume, sperm concentration, gross motility, individual motility and quality of motility following the protocols described by Soler et al. (2005) in order to assess the validity of the ejaculates for the experiment. Six observers estimated the parameters of sperm motility and the average value was recorded. The electroejaculation regime used was based on that employed previously for other ungulates by our group (Garde et al., 2003). Wave motility of the ejaculates of both ram was 4.5 in a score from 0 to 5, and the individual motility was 90% and 80% for the spermatozoa of rams #1 and #2, respectively. In order to obtain enough spermatozoa for the experiment, an equal number of cells from each ram (5000 x 10^6 spermatozoa) were mixed in 50 ml of incubation medium. The volume of ejaculates mixed in the final pool was 0.9 ml with a 5560 x 10^6 spermatozoa/ml of ram #1 and 2.3 ml with 2160 x 10^6 spermatozoa/ml of ram #2. The final concentration of the suspension used in the study was 200 x 10^6 spermatozoa/ml. The incubation medium was a Tyrode modified by Shi and Roldán (1995) by adding sodium pyruvate (1.0 mM) and sodium lactate at 60% (21.6 mM) without antibiotics (pH = 7.54, 290-300 mOsmol/kg). Pb acetate (Sigma-Aldrich, Steinheim, Germany) was previously incorporated in incubation medium to obtain three treatments with Pb spiked concentrations of 0, 50 and 500 ng/ml (0, 0.24 and 2.4 µM) 1and 12 aliquots of 1 ml of each treatment were incubated for 0, 1, 3 or 6 hr at 38.5 ºC (n = 3 for each treatment by incubation time). The basal concentration of Pb in the sperm suspension with incubation medium measured by graphite furnace-

atomic absorption spectrophotometry was 8.4 ng/ml, thus the experimental concentrations were 8.4, 58.4 and 508.4 ng/ml. The Pb concentrations used for the incubation covered the wide range described in sperm and seminal plasma of different species (Alexaki et al., 1990; Xu et al., 1993; El-Zohairy et al., 1996; Alexander et al., 1998; Benoff et al., 2003b; Pant et al., 2003) and a similar experimental study done with human sperm (Benoff et al., 2003a).

2.2. Sperm quality measurements

Three replicates of each treatment were examined immediately after each incubation time for individual sperm motility, quality of motility, viability, acrosome and plasma membrane integrities and spermatozoa morphology following the protocols described by Soler et al. (2003, 2005) in the lab of Biology of Reproduction (UCLM). The remaining volume of each sample (975 μl) was frozen at -80 °C to study the biomarkers of oxidative stress and the fatty acid (FA) composition in the lab of IREC.

The % individual sperm motility (SM) was assessed in a small drop of sperm suspension between a glass slide and a coverslip at 37 ºC under a phase-contrast microscope (magnification x400). The quality of motility (QM) was scored from 0 to 5 according to the speed and linearity of the movement together. The index of sperm motility (SMI) was calculated using the following equation: SMI=[SM+(QMx20)]/2 (Garde et al., 2003).

Plasma membrane functionality was assessed using a hypo-osmotic swelling test as described by Garde et al. (1998). This technique consisted of mixing 0.01 ml of diluted sperm samples with 0.1 ml of hypo-osmotic solution (sodium citrate, 100 mOsmol/kg) and incubating the mixture at room temperature for 30 min. The

samples were then fixed in 2% glutaraldehyde buffered solution and evaluated by phase-contrast microscopy at ×400. The sperm membrane was considered functional in cases where the sperm tail was coiled and the result was expressed as %.

Membrane viability or integrity was evaluated by using a nigrosin–eosin (NE) stain. This stain was prepared as described Tamuli and Watson (1994). The sperm suspension (5 µl) was mixed with the NE stain (10 µl) at 37 °C, incubated for 30 sec, smeared and dried on a warm plate at 37 °C. The samples were evaluated using bright field microscopy at ×400. Live spermatozoa remained unstained, while dead cells were dull pink. The % live spermatozoa was expressed as viability (%).

Acrosomal integrity was evaluated after a 1:10 dilution in 2% glutaraldehyde in 0.165 M cacodylate/HCl buffer (pH 7.3). The % spermatozoa with intact acrosomes (i.e., with normal apical ridges) was assessed by phase-contrast microscopy at ×400. The spermatozoa morphology was studied with 5 µl of each of the fixed samples with cacodylate and glutaraldehyde to count the number of spermatozoa with the following three abnormal forms: bent tail, isolated head and disintegrated head. Membrane functionality and viability, acrosome integrity and morphology were studied in 100 spermatozoa per sample.

2.3. Oxidative stress parameters

Thiobarbituric acid-reactive substances (TBARS) were measured as an estimate of lipid peroxidation using the method described by Aust (1985). Briefly, 0.5 ml of the thawed suspension was mixed with 1 ml of freshly prepared 15% trichloroacetic acid (Merck, Darmstadt, Germany), 0.375% thioarbituric acid (Alfa-Aesar, Karlsruhe, Germany) and hydrochloric acid 0.25 N (Panreac, Barcelona, Spain) and 1% butylated

hydroxytoluene (Panreac, Barcelona, Spain) in deionized water. The tubes were capped and heated for 30 min at 85 °C in a heatblock (VWR, USA). After this time the samples were cooled in ice to stop the reaction and were later centrifuged for 15 min at 3500 rpm with a centrifuge Meditronic BL (Selecta, Barcelona, Spain). The supernatant was transferred to a semi-micro plastic cuvette and the absorbance measured at 535 nm in a spectrophotometer Ultrospec 2100 Pr UV/Visible (Amersham Biosciences, Uppsala, Sweden). Standard curves were generated for the assay using 1, 1, 3, 3-tetramethoxypropane (Alfa-Aesar, Karlsruhe, Germany).

The activities of glutathione peroxidase (GPX; EC 1.11.1.9) and superoxide dismutase (SOD; EC 1.15.1.1) were recorded spectrophotometrically by micromethods using the Ransel and Ransod kits (Randox Laboratories, Crunlim, UK) with an automated analyzer A25 (BioSystems, Barcelona, Spain). GPX and SOD were determined based on the methods described by Paglia and Valentine (1967) and Woolliams et al. (1983), respectively.

For GPX, 200 µl of sperm suspension were diluted with 200 µl of Ransel diluting agent (Randox Laboratories, Crunlim, UK). In the reaction cell at 37 ºC, 220 µl of reagent 1 containing 4 mmol/l glutathione, ≥0.5 U/l glutathione reductase and 0.34 mmol/l NADPH diluted in 0.05 mol/l phosphate buffer, pH 7.2, with 4.3 mmol/l ethylenediamine tetraacetic acid were mixed with 5 µl of diluted sample and 15 sec later 10 µl of 0.18 mmol/l cumene hydroperoxide were added. The absorbance was read at 340 nm between 75 and 195 sec after sample addition and the enzyme activity was calculated by multiplying the increase of the absorbance per min by a factor of 8412. For SOD determination, 100 µl of sperm suspension was diluted with 300 µl of 0.01 mol/l phosphate buffer, pH 7. In the reaction cell at 37 ºC, 170 µl of mixed substrate containing 0.05 mmol/l xanthine and 0.025 mmol/l 2-(4-iodophenyl)-3-(4-nitrophenol)-

5-phenyltetrazolium chloride were mixed with 5 µl of diluted sample, and 15 sec later 25 µl of 80 U/l of xanthine oxidase were added. Mixed substrate was prepared in 40 mmol/l of N-cyclohexyl-3-aminopropanesulfonic acid buffer, pH 10.2, with 0.94 mmol/l ethylenediamine tetraacetic acid. The absorbance was read at 505 nm between 45 and 225 sec after sample addition and the kinetic of the enzyme activity was calculated based on a calibration curve performed with SOD standards at concentrations ranging from 0.192 to 4.6 U/ml in 0.01 mol/l phosphate buffer, pH 7. A reference control of bovine blood with 317 U/ml was analyzed after a 1:200 dilution and the obtained concentration was 318 U/ml.

2.4. Fatty acid composition

The composition of FA was analyzed as described in previous works to evaluate the effect of Pb on lipid metabolism (Mateo et al., 2003a). The suspension of spermatozoa (200 µl) was added to 1.8 g of anhydrous sodium sulphate (Panreac, Barcelona, Spain). After the addition of 10 µl of tridecanoic acid (13:0, 10 µg/µl in methanol) (Sigma, Steinheim, Germany) as internal standard and 2 ml of H_2SO_4 1N in methanol (Panreac, Barcelona, Spain), the headspace of the tubes was filled with pure N_2 to avoid oxidation, the content mixed with a vortex and the tubes placed in an oven at 80 ºC for 6 hr and at 60 ºC for 12 hr, with occasional shaking. FA methyl esters (FAMEs) were extracted with 1.2 ml of *n*-hexane (Panreac, Barcelona, Spain) after adding 2 ml deionized water. FAMEs were analyzed by gas chromatography (GC) coupled to electronic impact-mass spectrometry (EI-MS). The chromatographic system consisted in a 6890N Network GC System with a 5973 Network Mass Selective Detector (Agilent Technologies, Santa Clara, CA, USA). The capillary chromatographic

column used was a BPX70 (SGE, 30 m x 0.25mm I.D., 0.25 μm of film thickness, Cromlab, Barcelona, Spain). The injector (splitless mode) was at 270 ºC and the oven was maintained for 5 min at 100 ºC and then increased to 240 ºC at a ramp rate of 2.5 ºC/min. The carrier gas was helium at a flow of 1.2 ml/min. The source of the mass spectrometer was at 230 ºC and the voltage was 70 volts. The identification and quantification was achieved by comparison to retention times of FAME reference standards (FAMQ-005, AccuStandard, New Haven, CT, USA) and the mass spectra. Results were expressed as % of individual FAMEs from the total amount.

3. Statistical analysis.

The effects of Pb concentration and incubation time on the seminal parameters and the oxidative stress biomarkers were studied with two-way analyses of variance (ANOVA). The % PUFA and other individual FA were also studied with two-way ANOVA tests. Comparisons of the different parameters among Pb-treatments for each incubation time were studied with least significant differences tests. The effects on the FA profile were studied by means a compositional analysis because the sum of FAME is always 100 and variations in an individual FA imply changes in the values of the rest (Mateo et al., 2003a). The % each FA was log-transformed and the ratio with the stearic acid (18:0) was calculated. A principal component analysis was performed with the obtained log-ratios and the PC1 calculated, which summarized 46.2% of total variance, was used as the other variables in a two-way ANOVA to explore differences in FA composition due to the presence of Pb and the incubation time. As the % stearic acid was found to be affected by the incubation time, the compositional analysis was also performed by using palmitic acid (16:0) for the calculation of the log-ratios to evaluate

changes apart from stearic acid fluctuations. PC1 obtained in this analysis summarized 47.4% of the total variance. The criterium for significance was set at $p < 0.05$. Statistics were performed with the SPSS 13.0 program.

4. Results.

4.1. Parameters of sperm quality

Sperm motility was significantly reduced by Pb concentration in the incubation medium and by incubation time. The interaction between both factors (time and Pb concentration) was significant. The effect of Pb on sperm motility was more marked after 3 hr of incubation at the highest Pb concentration (Figure 1a). The quality of motility was markedly reduced by Pb and time-dependent. The interaction of both factors was also significant. The quality of motility decreased at the onset of incubation with 500 ng/ml of Pb and after 1 hr with 50 ng/ml (Fig 1b). Sperm motility index (SMI) was significantly reduced by Pb and incubation time-dependent. The interaction of both factors was significant (Table 1).

The membrane functionality evaluated by the hypo-osmotic swelling test was markedly reduced by Pb and time-dependent. This reduction was similar at 50 and 500 ng/ml of Pb concentration after 1-3 hr of incubation (Figure 1c). The interaction between factors was not significant. Membrane integrity (viability) was markedly reduced by Pb and incubation time-dependent. This decrease of sperm viability was only significant after 6 hr incubation with the highest Pb concentration (Figure 1d). The interaction between factors was not significant. Acrosomal integrity was markedly reduced by Pb concentration in a time-dependent manner. The interaction between both

factors was also significant. This parameter showed a Pb concentration-response relationship, especially at the onset of the incubation and 1 hr after incubation (Figure 1e). Only tail abnormalities (bent tail) were related to the presence of Pb in the incubation medium and time-dependent. The effect of Pb was significant at the highest Pb concentration after 3 hr incubation (Figure 1f). The interaction between factors was not significant.

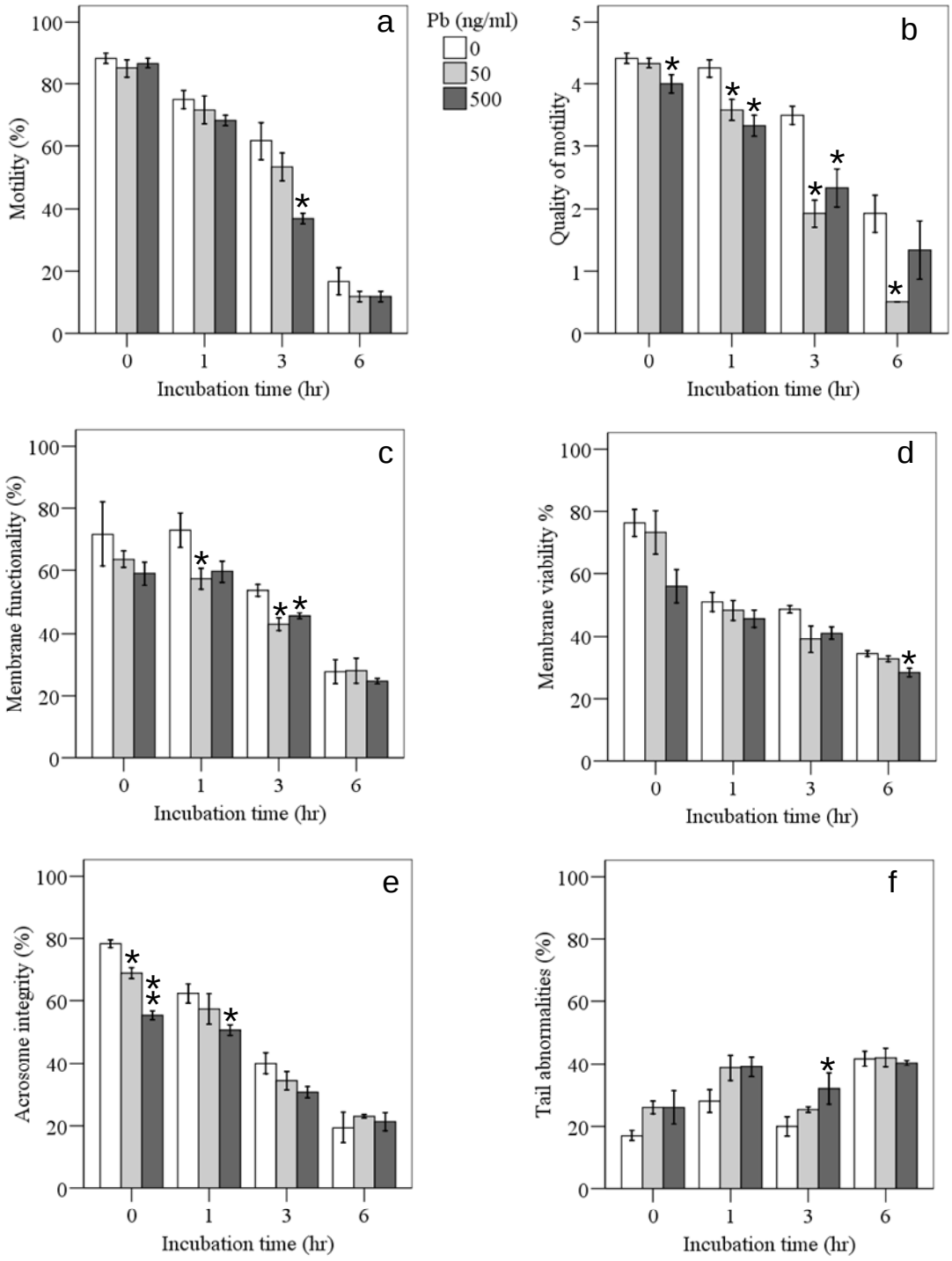

Fig. 1. Sperm motility (a), quality of motility (b), membrane functionality (c), and viability (d), acrosome integrity (e) and tail abnormalities (f) observed in ram spermatozoa spiked with 0, 50 or 500 ng/ml of lead and incubated during 0, 1, 3 or 6 hr (means with S.E. bars). All these parameters have been affected by the presence of Pb and the incubation time. *Significantly different from controls without Pb at each incubation time and **significantly different from controls and 50 ng/ml Pb-treated samples (least significant differences test, $p < 0.05$).

Table 1. Index of Sperm Motility (mean ± SD) in Ram Sperm Incubated at 38,5 ºC with Three Concentrations of Pb.

[Pb] (ng/ml)	Incubation time (hr)			
	0	1	3	6
0	88 ± 3	80 ± 4	66 ± 7	28 ± 9
50	86 ± 4	72 ± 6	46 ± 8*	11 ± 1*
500	83 ± 4	67 ± 4*	42 ± 6*	19 ± 8

*Significantly different from controls without Pb at each incubation time (least significant differences test, $p < 0.05$). N=3 replicates for each treatment

4.2. Oxidative stress parameters and fatty acids composition

The activity of GPX was not affected by Pb, but decreased significantly with incubation time (Table 2). The activity of SOD was not affected by Pb or the incubation time (Table 2). Lipid peroxides measured as TBARS were significantly lower in the samples incubated with Pb and this difference decreased with incubation time.

GPx (mU/ml), SOD activity (UI/ml), and TBARS (nmol/ml) (mean ± SD) in Ram sperm Incubated at 38,5 ºC with three concentrations of Pb.

Table 2. GPx (mU/ml), SOD activity (UI/ml), and TBARS (nmol/ml) (mean ± SD) in Ram Sperm Incubated at 38,5 ºC with Three Concentrations of Pb.

[Pb] (ng/ml)	Biomarker	Incubation time (hr)			
		0	1	3	6
0	GPx	564 ± 89	491 ± 45	486 ± 69	452 ± 56
	SOD	4.31 ± 0.34	4.61 ± 0.28	4.40 ± 0.50	4.93 ± 0.11
	TBARS	2.14 ± 0.63	1.46 ± 0.13	1.33 ± 0.09	1.34 ± 0.10
50	GPx	537 ± 48	573 ± 25	489 ± 36	447 ± 17
	SOD	4.67 ± 0.48	4.59 ± 0.04	4.57 ± 0.48	4.74 ± 0.43
	TBARS	1.27 ± 0.11*	1.16 ±0.12*	1.33±0.10	1.16 ±0.25
500	GPx	519 ± 36	483 ± 24	468 ± 26	529 ± 56
	SOD	4.22 ± 0.10	4.11 ± 0.78	4.43 ± 0.40	4.96 ± 0.56
	TBARS	1.43 ± 0.20	1.20 ±0.09*	1.26 ±0.06	1.19 ±0.08

*Significantly different from controls without Pb at each incubation time (least significant differences test, $p < 0.05$). N=3 replicates for each treatment.

The FA composition revealed a predominance of the polyunsaturated FA (45.2%) followed by unsaturated FA (40.2%, in samples without added Pb at the onset of the incubation) (Table 3). The % total PUFA, the most susceptible to oxidation, was not affected by Pb or the incubation time (Fig. 2) in agreement with the results obtained from TBARS measurement. The % 18:0 increased with the incubation time from 11.6% to 16.6% (Table 3). The PC1 of the log-ratios obtained from % each FA with 18:0 reflected the change in the overall composition due to the variation of 18:0 during the incubation time (Fig. 3). This PC1 is positively correlated with PUFA and more specifically with the % 22:6 n-3 (docosahexaenoic acid, DHA). However, this effect on the overall FA composition was not observed when log-ratios were calculated with 16:0 instead of 18:0, which means that the variation observed in FA composition was mainly

due to changes in % 18:0. In any of both compositional analyses there was a significant effect of Pb concentration on FA composition.

Table 3. Composition Fatty Acid (mean ± SD of %) of Ram Sperm at Different Incubation Times.

Fatty acid	Incubation time (hr)			
	0	1	3	6
14:0	4.51±0.57	4.36±0.40	3.34±0.52	4.10±0.36
DMAHD[a]	11.85±0.44	12.63±0.70	10.45±0.52	12.42±0.29
HD[b]	1.31±0.32	1.31±0.12	1.09±0.13	1.12±0.23
16:0	24.84±4.50	24.20±2.66	20.32±3.02	24.49±1.83
18:0	10.88±0.89	13.79±0.88*	15.29±1.02*	16.44±0.49*
18:1n-9	0.90±0.06	1.14±0.29	1.02±0.17	0.87±0.10
18:1n-7	0.54±0.17	0.27±0.09	0.34±0.13	0.26±0.08
18:2n-6	1.58±0.15	1.72±0.31	1.61±0.24	1.34±0.13
20:4n-6	1.31±0.30	0.74±0.27	0.75±0.28	0.78±0.13
22:5n-6	10.07±1.17	9.65±0.94	13.19±1.40	9.07±0.46
22:6n-3	32.20±4.92	30.19±3.25	32.60±5.18	29.10±1.55

[a]Dimethyl acetal of hexadecan-1-al, [b]*n*-hexadecanal. *Significantly different from the onset of incubation (least significant differences test, $p < 0.05$). N = 3 replicates for each treatment.

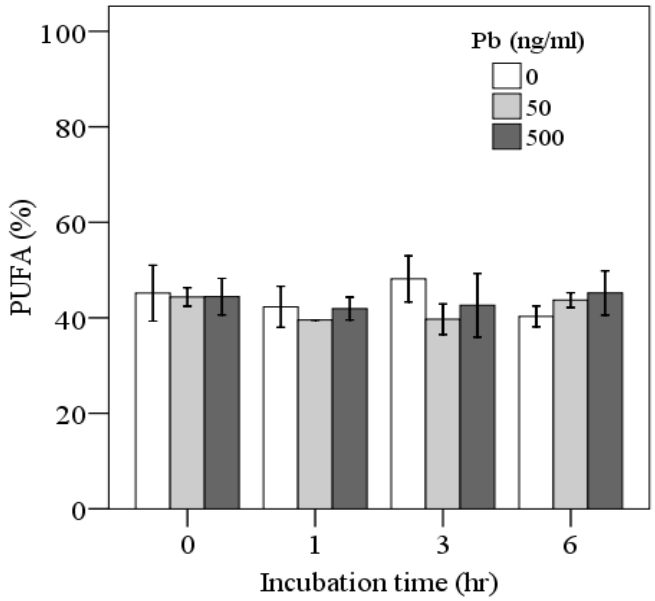

Fig. 2. Percentage of polyunsaturated fatty acids (PUFA) in ram spermatozoa spiked with 0, 50 or 500 ng/ml of lead and incubated during 0, 1, 3 or 6 hr (means with S.E. bars). The % PUFA was not affected by the presence of Pb or incubation time.

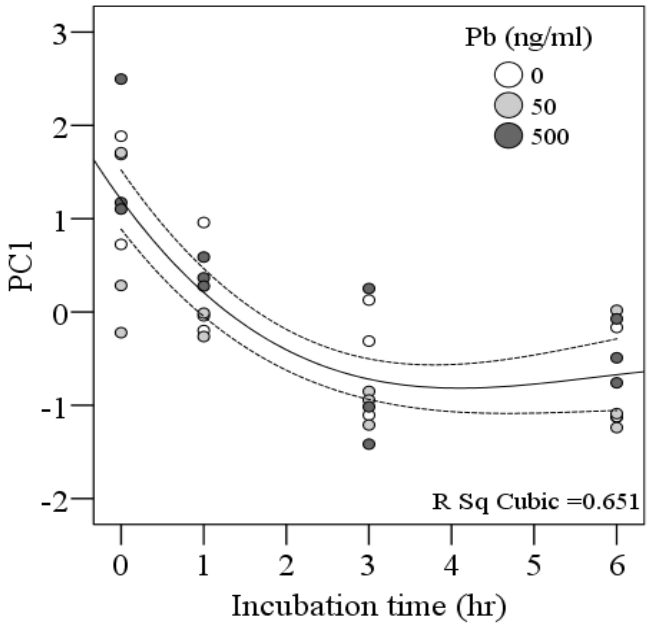

Fig. 3. Variation in the first component obtained from the principal component analysis of the log-ratios of % each fatty acids with 18:0 in ram spermatozoa spiked with 0, 50 or 500 ng/ml of lead and incubated during 0, 1, 3 or 6 hr. The composition of fatty acids was affected by incubation time, but not presence of Pb.

5. Discussion.

All the measurements of sperm quality, independently of the effect of the incubation time, were affected by the presence of Pb in the medium. The lack of effects on SOD and GPX activities, lipid peroxidation or % PUFA did not support the hypothesis of oxidative stress as the cause of the loss of sperm quality after *in vitro* exposure to Pb. However, other oxidative changes on membrane proteins or DNA have not been evaluated here and oxidative stress may have developed *in vivo* due to the effects of Pb on the antioxidant status (Hsu and Guo, 2002).

Other studies of spermatozoa exposure to Pb *in vitro* revealed similar effects on the sperm quality of other species. Sperm of rabbits in a suspension of 20-40 x 10^6 spermatozoa/ml and incubated at 37 ºC for 30 min in a medium with 0.025 mM of Pb (5170 ng/ml) produced a decrease in the fertilization rate (Foote, 1999). Bull sperm incubated at 20 ºC during 60 min with 2500 ng/ml of Pb showed a decrease in the sperm motility but no effect was observed at 250 ng/ml (Alexaki et al., 1990). Human sperm without any previous dilution incubated with an extremely high Pb concentration (50 µg/ml) showed a loss of motility that was not observed at lower concentrations (0.5 and 5 µg/ml) (Huang et al., 2001). However, in the present study Pb concentrations closer to the range of the expected Pb seminal concentrations were used (Alexaki et al., 1990; Xu et al., 1993; El-Zohairy et al., 1996; Alexander et al., 1998; Benoff et al., 2003b; Pant et al., 2003). Benoff et al. (2003a) exposed motile sperm from fertile donors in suspension of 12 x 10^6 spermatozoa/ml in capacitating media to increasing Pb concentrations (51.5, 518 and 5180 ng/ml) during overnight incubation and observed a reduced sperm motility and increased spontaneous acrosome reaction in a dose-dependent manner. The two lowest Pb concentrations used by Benoff et al. (2003a) are comparable to the doses

used in our experiment and the results obtained were very similar. The acrosome reaction induced by Pb is also a common finding in experimental and epidemiological studies. Johansson (1989) observed a reduced ability of spermatozoa from Pb-exposed mice to fertilize oocytes due to a premature acrosome reaction. Benoff et al. (2003b) observed Pb concentrations in human seminal plasma of 0.1-1.5 µg/ml which correlated with increased spontaneous acrosome reaction and reduced fertilization after artificial insemination.

Oxidative stress and excessive generation of ROS in sperm has been associated with male infertility (Rao et al., 1989; de Lamirande and Gagnon, 1992; Sikka, 1996; Aitken, 2006). Pb promotes oxidative stress by different mechanisms (Hsu and Guo, 2002) and several studies associated the effects of Pb on sperm with excessive production of ROS, lipid peroxidation or inhibition of antioxidant enzymes. Male rats injected intraperitoneally with Pb at a dose of 10 mg of Pb acetate/Kg/week for 6 or 9 weeks showed a significant correlation between blood (336-480 ng/ml) and sperm concentrations (670-888 ng/10^9 spermatozoa), lower epididymal sperm counts and motility, and higher ROS formation accompanied with a reduced sperm-oocyte penetration rate (Hsu et al., 1997). Such effects were reduced by the supplementation of rats with vitamin E and C suggesting a role of oxidative stress in the effects of Pb on sperm (Hsu et al., 1998b). In another experiment with male rats exposed to Pb, Hsu et al. (1998a) observed a reduction of serum testosterone and an increase of capacitated and acrosome-reacted spermatozoa and ROS formation. Marchlewicz et al. (2004) also observed an increased formation of ROS in testes and epididymis of rats, which was reduced with the dietary supplementation with vitamin C. Huang et al. (2001), with sperm incubated *in vitro* with much higher Pb concentrations, did not observe an increase in lipid peroxidation. Xu et al. (2003) observed a positive correlation between

oxidative DNA damage in human spermatozoa and Pb concentration in seminal plasma, although this relationship was more significant for cadmium.

The activities of the antioxidant enzymes studied here were not affected by Pb. In contrast, several studies found Pb-induced alterations in these enzymatic activities in tissues like liver, blood, kidney and brain (Gelman et al., 1979; Sandhir et al., 1994; Dagget et al., 1998; Adonaylo and Oteiza, 1999; Hunaiti and Soud, 2000; Mateo et al., 2003b).

The presence of *n*-hexadecanal and dimethyl acetal of hexadecan-1-al is explained by the abundance of plasmalogens in ram spermatozoa (Scott et al., 1967). Plasmalogens are a special group of glycerophospholipids in which the sn-1 position of the glycerol backbone is linked with a long chain fatty aldehyde (n-hexadecanal or n-octadecanal) via a vinyl-ether bond. The vinyl-ether bond of plasmalogens may be among the first targets of free radicals attack conferring antioxidant properties to these molecules (Brites et al., 2004). On the other hand, the most abundant FA is highly polyunsaturated (22:6n-3) and susceptible to oxidation, thus plasmalogens may have had an important role to maintain FA composition during the incubation of sperm in our experiment.

One of the effects of Pb observed here, the acrosome reaction, may be explained by other mechanisms unrelated to oxidative stress. Genetic polymorphisms in voltage-gated K^+ channels (VGKC) was associated with variations in the susceptibility to Pb (Benoff, 1999). These channels are upstream of the L-type voltage-dependent Ca^{2+} channels (L-VDCC) which regulates the sustained Ca^{2+} influx required for the acrosome reaction (Benoff, 1999). Evidence exists that a VGKC capable of transporting Pb is expressed on the human sperm head and that specific inhibitors of such channels (i.e.

charybdotoxin) reduce the effect of Pb on spontaneous acrosome reaction (Benoff et al., 2000).

In summary, data verified that *in vitro* several of the effects of Pb on sperm quality usually observed *in vivo* also occurred, but at lower concentrations than in other *in vitro* experimental studies and without any relationship with oxidative stress biomarkers or significant changes in the fatty acid composition. The ram spermatozoa seem to be especially sensitive to Pb and this may be useful for the study of the mechanisms of Pb on sperm function.

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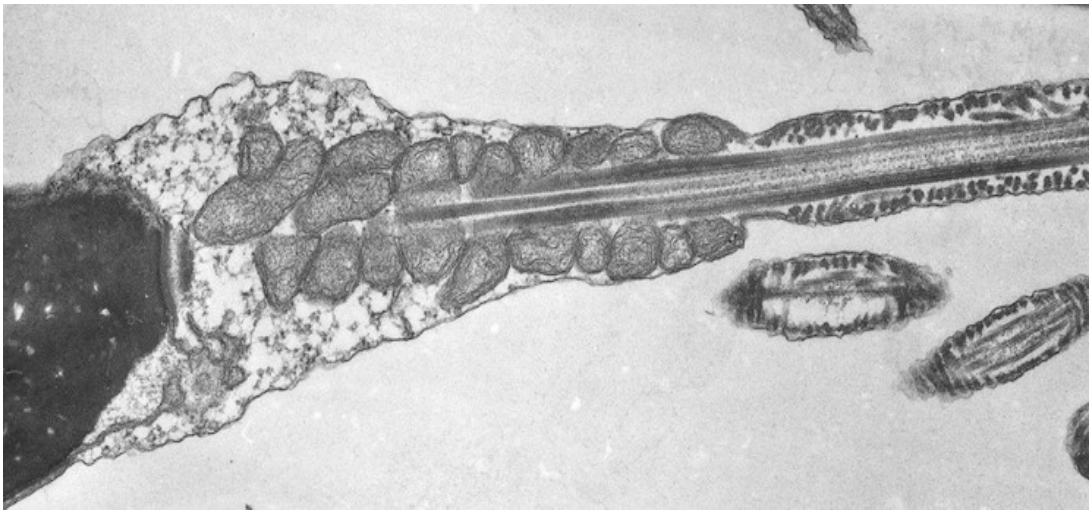
CAPÍTULO 6

Effects of capacitation inhibitors on lead-induced changes in ram spermatozoa



Pieza media del espermatozoide con mitocondrias alrededor de la estructura de microtúbulos del flagelo (TEM Microscopio electrónico de transmisión)

Castellanos P, Maroto-Morales A, García-Álvarez O, Garde JJ, Mateo R. 2014. (En preparación).

Efectos de inhibidores de la capacitación sobre los cambios inducidos por plomo en espermatozoide de morueco

RESUMEN

El Plomo (Pb^{2+}) es un metal pesado tóxico que interfiere con varios procesos fisiológicos regulados por Ca^{2+}, incluyendo el proceso de capacitación del espermatozoide que se caracteriza por cambios de la membrana y la motilidad del espermatozoide necesaria para la fertilización del ovocito. En este estudio, los esperamtozoides eyaculados de seis moruecos (*Ovies aries*) han sido incubados *in Vitro* con o sin 50 ng Pb^{2+}/ml durante 30 minutos y con o sin tres diferentes moduladores potenciales de los efectos del Pb^{2+} en los espermatozoides: charidobtoxina, quinacrina y estaurosporina. Las muestras incubadas con Pb^{2+} han mostrado reducciones significativas en la integridad del acrosoma y en la viabilidad de los espermatozoides. Ninguno de los tres inhibidores estudiados ha tenido un efecto protector contra el Pb^{2+}. Por el contrario, los espermatozoides incubados con plomo tuvieron una menor integridad de acrosoma cuando éstos se incubaron con estaurosporina e igualmente la viabilidad de los espermatozoides fue inferior cuando se incubaron con charidobtoxina. La quinacrina fue la única sustancia capaz de aumentar la concentración de Pb^{2+} en los espermatozoides, por lo tanto la mejoría en los efectos del Pb^{2+} por el tratamiento con estaurosporina y charidobtoxina no se ha producido por el aumento en la captación de Pb^{2+} por los espermatozoides.

***Palabras clave*:** capacitación espermática, integridad del acrosoma, niveles de calcio, metales pesados, canales de membrana.

ABSTRACT

Lead (Pb^{2+}) is a toxic heavy metal which interferes with several physiological processes regulated by Ca^{2+}, including the sperm capacitation process characterized by changes of the membrane and the motility of spermatozoa necessary for the fertilization of the oocyte. In this study, ejaculated sperm of six ram (*Ovis aries*) have been incubated *in vitro* with or without 50 ng Pb^{2+}/ml during 30 minutes and with or without three different potential modulators of the effects of Pb^{2+} on spermatozoa: charybdotoxin, quinacrine and staurosporine. Sperm samples incubated with Pb^{2+} have shown significant reductions in acrosome integrity and sperm viability. None of the studied blockers had a protective effect against Pb^{2+}. On the contrary, Pb-incubated sperm had lower acrosome integrity when it was also incubated with staurosporine and lower sperm viability when it was incubated with charybdotoxin. Quinacrine was the only substance capable of increasing the concentration of Pb^{2+} in spermatozoa, thus the enhancement of Pb^{2+} effects produced by staurosporin and charybdotoxin were not produced by an increased uptake of Pb2+ by spermatozoa.

Keywords: sperm capacitation, acrosome integrity, calcium levels, heavy metals, membrane channels.

1. Introduction.

The membrane of spermatozoa must remain stable during spermatogenesis, sperm maturation and storage, but a process that affects the physiology of the sperm membrane is triggered during the fertilization to produce the exocytosis of the secretory granule known as acrosome and the fusion of the sperm cell itself with the oocyte. This process known as capacitation takes place in the female reproductive tract, but it can also be induced *in vitro* by incubation of the spermatozoa with specially developed media. During this capacitation process there is an intracellular increase of Ca^{2+} level and pH facilitated by the contact with uterine secretions (Lishko et al., 2010). Changes that occur in the spermatozoa membrane start with the cholesterol efflux to increase membrane fluidity, followed by changes in the transport of ions that produce a hyperpolarization and an increase in protein tyrosine phosphorylation by activation of a cAMP-dependent pathway (Demarco et al., 2003; Visconti et al., 2002, 2011). This hyperpolarization process would in turn increase Ca^{2+} uptake through Ca^{2+} permeable channels, some of which are activated by alkalinization (Visconti et al., 2011), and finally leading to the post-capacitation event named as acrosome reaction (Flormann et al., 2008; De la Vega-Beltrán et al., 2012) (*Fig. 1*).

Heavy metal ions are potent environmental toxicants and specifically lead (Pb^{2+}) is known to interfere with a range of physiological process regulated by Ca^{2+} (Golstein, 1993; Evans et al., 2003; Chen et al., 2009), which leads to explain the interaction of Pb^{2+} in cytosolic pathways and mechanisms involved in the capacitation process of spermatozoa. Among these, Pb^{2+} has extra- and intracellular effects by interaction with a number of Ca^{2+} dependent effectors such a calmodulin (a Ca^{2+} receptor protein) and Ca^{2+}-dependent K channels in the plasma membrane (Schanne et al., 1997; Sun et al.,

1999), but also on enzymes such as protein kinase C (PKC) (Murakami et al., 1993; Long et al., 1994). PKC has been identified as a cellular target for Pb^{2+} with different effects depending on the concentration. At pM or nM concentrations of Pb^{2+} there is an activation of PKC through the interaction with Ca^{2+} (Long et al., 1994; Sun et al., 1999), while at µM concentrations Pb^{2+} inhibits the kinase activity by affinity with its catalytic domain (Sun et al., 1999). Furthermore, Pb^{2+} may exert its effect through the activation of phospholipases. Phospholipase A (PLA) releases arachidonic acid (ArA), which is used by lipoxygenase to produce 15-hydroxyl-5,8,11,13-eicosatetraenoic acid (15-HETE) and prostaglandins (PGE) involved in the process of acrosome reaction and sperm capacitation (Lax et al., 1990; Breitbart et al., 1995). In vascular cells, 1 µM Pb^{2+} can induce PGE secretion by upregulation of the transcription of COX-2/cPLA genes (Chang et al., 2011). Finally, oxidative stress induced by Pb^{2+} through the depletion of reduced thiols or inhibition of antioxidant enzymes (Reglero et al., 2009a, 2009b) could also induce changes in membrane permeability due to the particular composition of the sperm membrane with high amount of easily oxidizable polyunsaturated fatty acids (PUFA) (Delbarco-Trillo and Roldán, 2013) (*Fig. 1*).

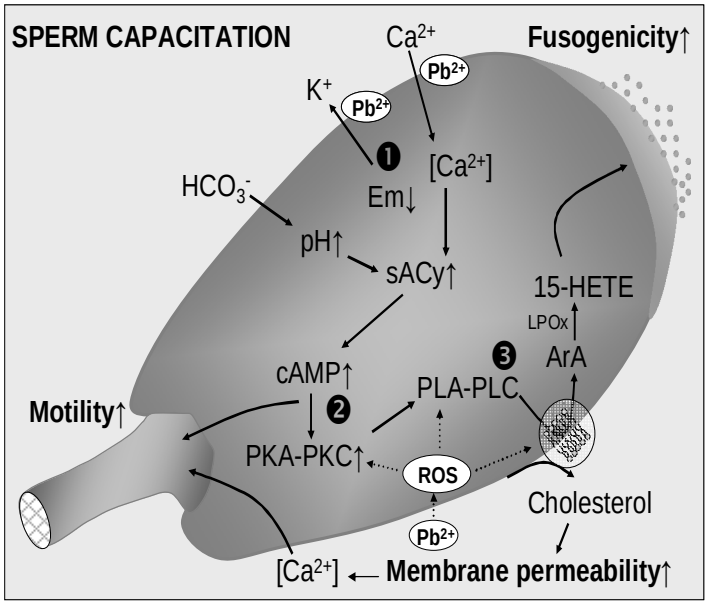


Fig.1. Scheme of the capacitation in the spermatozoa. This figure illustrates a schematic representation of the interrelationship between the ions channels involved in the capacitation process and the routes in which the inhibitors charydobtoxin (1), staurosporine (2), quinacrine (3) and Pb^{2+} could intervene in this process. Capacitation starts with an increase of motility in the tail sperm and ends with the loss of acrosome membrane integrity and the enzymes release thereof. Bicarbonate is present at low concentration in epididymal and ejaculated sperm suspension and enters into the sperm cell to contribute together with an increase of Ca^{2+} uptake through Ca^{2+} permeable channels to the cytoplasm alkalinization (pH↑) and membrane hiperpolarization (Em↓) (Demarco et al., 2003; Visconti et al., 2011). These changes in the transport of K^+ (voltage gated K^+ channels, VGK^+C) and Ca^{2+} activates soluble adenylil ciclase (sACy) which through the activation of protein tyrosine kinase A (PKA) and protein tyrosine kinase C (PKC) induced by the increase in the cyclic adenosine (cAMP) allows an increase in protein tyrosine phosphorilation and the activation of flagellar movement (Visconti et al., 2002; Visconti et al., 2011). These changes were initiated by the cholesterol efflux from the sperm membrane that increase the membrane permeability and fluidity needed for fusogenity (Flormann et al., 2008; De la Vega-Beltrán et al., 2012) and acrosme enzymes release. In this increase of membrane permeability also contributes the phospholipase A (PLA) and phospholipase C (PLC), activated by PKC leading to a release from membrane of arachidonic acid (ArA) that transforms into 15-hidroxyleicosatetraenoic acid (15-HETE) by the lypoxigenase enzyme (LPOx) and contributes to complete the process (Breitbart et al., 1995). All these changes driven by Ca^{2+} uptake can be affected by competition of Pb^{2+} on VGK^+C and Ca^{+2} channels, or directly by Pb^{2+} effects on the antioxidant defence which derives in membrane lipoperoxidation.

In previous studies, we have found negative effects of Pb2+ on the sperm viability, acrosome integrity and changes in the fatty acid composition of spermatozoa collected form the epididymis of red deer (*Cervus elaphus*) living in a Pb-mined area (Reglero et al., 2009a; Castellanos et al., 2010). Moreover, some of the adverse effects of lead on spermatozoa, like premature acrosome reaction and reduced cell viability, may occur after epididymal sperm maturation when it contacts seminal plasma or the female genital tract, as *in vitro* experiments have shown (Benoff et al., 2003;

Castellanos et al., 2008). Here we have studied the potential mechanisms by which Pb^{2+} can exert its action on membrane viability and acrosome integrity. For this purpose we have used charybobtoxin as a voltage-gated potassium channels ($VGK^{+}C$) blocker (Sun and Suszkiw, 1994), staurosporine as a potent PKC inhibitor affecting on cytosolic calcium homeostasis (Morgan et al., 2007) and quinacrine as a non-selective monoamine oxidase A /B (MAO A/B) inhibitor of $cPLA_2$ and the subsequent synthesis of 15-HETE (15- hidroxileicosatetraenoic acid) and prostaglandins (Lee et al., 2009). We hypothesised that these inhibitors of $VGK^{+}C$, PKC and $cPLA_2$ could modulate the observed effects of Pb on the acrosome integrity and membrane viability of spermatozoa.

2. Material and methods.

2.1. Semen collection

Semen of six healthy rams (Ovis aries) of Manchega breed was collected by artificial vagina during the scheduled sperm collection in the Animal Reproduction Centre (CERSYRA, Valdepeñas). Sperm is routinely collected in the Manchega males of this Centre for the artificial insemination of the herds of Castilla-La Mancha region. Therefore, these males have been previously evaluated and selected by their quality as donors. Two or three ejaculates per animal were collected and the one with the best wave motion was used for the experiment. Volume, sperm concentration, wave motion, individual sperm motility, quality of movement, acrosome integrity and sperm viability were recorded (Table 1). Sperm concentration was determined by transmittance with a spectrophotometer (Type Helios Delta model, Thermo Electron Corporation), diluting 5

µl of ejaculates in 5 ml of sodium citrate 128 nM. Wave motion was subjectively scored from 1 to 5, where 0 is no movement and 5 is strong wave movement, on a wet mount of neat semen using bright field microscopy at ×10 (BH-2 Olympus). Individual sperm motility and quality of movement were assessed with 5 µl of semen diluted on 200 µl of phosphate buffer-saline (PBS) and after incubation at 37 ºC for 5 min., 5 µl of sperm diluted were placed between a pre-warmed slide and a 22 mm × 22 mm coverslip and were observed at ×400 under phase-contrast optics (Eclipse 50i Nikon). The percentage of motile sperm was estimated subjectively by three experienced observers and was determined by analysing four or five fields of view (Evans and Maxwell 1987) under the microscope with values ranging from 0% when no motile spermatozoa were observed, to 100% when all spermatozoa were moving. Quality of sperm movement was also estimated subjectively on a scale 0-5 where 0 is no motility and 5 is vigorous progressive movement. Acrosomal integrity was evaluated with 10 µl of each sample diluted with 50 µl of 2% glutaraldehyde in 0.165 M cacodylate/HCl buffer (pH 7.3). The % spermatozoa with intact acrosomes (i.e., with normal apical ridges) was assessed at ×400 under phase-contrast optics. Sperm viability was evaluated by means of nigrosin–eosin stain as described Tamuli and Watson (1994). The diluted sperm (5 µl) was mixed with the nigrosin–eosin stain (10 µl) at 37 °C, incubated for 30 sec, smeared and dried on a warm plate at 37 °C. The samples were evaluated using bright field microscopy at ×400. Live spermatozoa remained unstained, while dead cells were dull pink. The % live spermatozoa were expressed as viability (%). Acrosome integrity and sperm viability and were assessed in 100 spermatozoa per sample.

Table 1. Initial values before incubation of the ejaculates obtained from six rams used as donors for the in vitro experiment.

Ram #	Volume (ml)	Concentration (10^6 spz/ml)	Wave motion	Motility (%)	Quality of movement	Acrosome integrity (%)	Viability (%)*
1	2.0	4,920	3.75	65	4	74	84
2	1.0	5,000	3.5	80	4.5	65	87
3	1.0	4,730	3.75	40	4	67	75
4	0.5	3,900	4	85	4.5	67	86
5	1.3	3,500	3	70	4	57	81
6	1.8	4,250	3.25	70	3.25	64	86

*Values of the control samples after 30 min incubation

2.2. Treatments of spermatozoa

In order to obtain a suspension of 300 × 10^6 spermatozoa/ml of each animal, the corresponding volume was diluted according to the concentration of spermatozoa of each ejaculate. Such volumes were diluted with BGM-3 medium (pH 7.58 and osmolality 305 mOsm/kg), which is a modified tyrode medium supplemented with the 10 mM sodium pyruvate (Sigma Ultra 99%) and 6 mg/ml of bovine serum albumin (BSA) (albumin bovine serum, factor V 96% Sigma).

Finally, sperm suspensions of each ram with 150 × 106 spermatozoa/ml were distributed in eight aliquots and assigned to the following treatments to be incubated 30 min at 37 ºC: (1) control, (2) 50 nM charybdotoxin, (3) 0.1 nM staurosporine, (4) 50 µM quinacrine (5) 50 ng/ml (0.24 µM) Pb^{2+}, (6) 50 ng/ml Pb^{2+} + 50 nM charybdotoxin, (7) 50 ng/ml Pb^{2+} + 0.1 nM staurosporine, and (8): 50 ng/ml Pb^{2+} + 50 µM quinacrine. Pb^{2+} solution was prepared from lead II acetate tri-hydrate (99%, Sigma-Aldrich).

Charybdotoxin (Sigma-Aldrich) was initially prepared in a solution at 20 μM in 150 mM NaCl. Staurosporine from *Streptomyces* sp. (98%, Sigma-Aldrich) was prepared in an initial solution at 200 μM in DMSO (2%). Quinacrine dihydrochloride (90%, Sigma-Aldrich) was initially prepared in a solution at 5 mM in hot water. All the subsequent dilutions to obtain the working solutions of these chemicals were prepared in BGM-3 medium.

Pb^{2+} concentration and incubation time were selected on basis to previous studies that permitted to find the optimal *in vitro* conditions to obtain effects on sperm quality parameters at low Pb^{2+} levels (Castellanos et al., 2013). The concentrations of the capacitation inhibitors applied in the incubation medium were those used in similar *in vitro* studies with sperm with charybdotoxin (Benoff et al. 2000), staurosporine and quinacrine (Breitbart et al., 1995).

2.3. Analysis of sperm quality parameters and Pb concentration in spermatozoa

After 30 min of incubation at 37 ºC, individual sperm motility, quality of movement, acrosome integrity and sperm viability were measured in 20 μl of each aliquot as described above. The remaining 980 μl after incubation of the eight treatments of sperm suspensions corresponding to each of the six males (n=48) were centrifuged at 940 rcf for 10 min in a tared eppendorf tube and the supernatant was decanted. The mass of the pellet was calculated and it was stored at -20 ºC for Pb analysis. Lead concentration in spermatozoa was analysed by graphite furnace atomic absorption spectroscopy system (AAnalyst 800, Perkin Elmer) as described in Castellanos et al. (2013). All concentrations were given in dry weight (d.w.).

2.4 Statistical analysis

The parameters of sperm quality and Pb concentration in spermatozoa after incubation were compared among treatments with generalized linear models (GLM) including the animal (donor ram), the Pb treatment (0 or 50 ng/ml of Pb^{2+}) and the inhibitor used (none, charybdotoxin, staurosporine or quinacrine) as predictors. Marginal means obtained with these models were compared to find differences between the different pair of combinations of Pb and inhibitors. The significance of the statistics was set at $p \leq 0.05$. The statistical analysis was performed with IBM SPSS Statistics v. 19.0.0.

3. Results.

Motility of spermatozoa was not affected by the incubation with Pb or the potential inhibitors of the capacitation process (Table 2). Motility was dependent on the donor individual (Wald's $\chi 25 = 483$, $p<0.001$). The quality of movement differed between spermatozoa treated with or without Pb (Wald's $\chi 21 = 6.25$, $p=0.012$) and among the different treatments with potential inhibitors (Wald's $\chi 23 = 10.25$, $p=0.017$).

The quality of movement was higher in the spermatozoa treated staurosporine, quinacrine and charydbotoxin, and especially in those incubated with Pb (Table 2).

The quality of the movement was also highly dependent of the donor (Wald's $\chi 25 = 53$, $p<0.001$).

The acrosome integrity was significantly reduced by incubation with Pb (Wald's $\chi^2_1 = 5.57$, $p=0.018$) and the different inhibitors (Wald's $\chi^2_3 = 15.92$, $p<0.001$). Acrosome integrity was also dependent on the donor (Wald's $\chi^2_5 = 90.2$, $p<0.001$). The

lowest percentage of intact acrosomes was observed in the group treated with Pb + staurosporine.

Sperm viability was affected by the incubation with Pb (Wald's $\chi 21 = 4.92$, $p=0.027$), but not by the potential inhibitors ($p=0.498$). Spermatozoa viability was less dependent on the donor (Wald's $\chi 25 = 10.99$, $p=0.052$). The comparison of marginal means showed that spermatozoa incubated with Pb and charybdotoxin had a lower sperm viability than those incubated without Pb or only with quinacrine (Table 2).

The concentration of Pb in spermatozoa was significantly affected by the presence of Pb in the incubation media (Wald's $\chi^2_3 = 505.5$, $p<0.001$), the potential inhibitors added (Wald's $\chi^2_3 = 10.2$, $p=0.017$) and the interaction between these two factors (Wald's $\chi^2_3 = 10.2$, $p=0.017$). This interaction reveals that Pb concentration in Pb-exposed spermatozoa was higher in those incubated with quinacrine than in the other spermatozoa incubated with Pb (Table 2). In this case, the effect of the donor was not significant ($p=0.087$)**.**

Table 2. Effects on ram spermatozoa of the incubation with Pb^{2+} (50 ng/ml) and three different potential blockers of the changes induced by this metal.

Treatment	Motility (%)		Quality of movement (0-5)		Acrosome integrity (%)		Sperm viability (%)	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Control	70.0	5.3	3.71^{a}	0.12	37.8^{a}	3.1	83.2^{a}	1.8
Charybdotoxin	69.2	6.0	3.83^{ab}	0.11	32.8^{bc}	3.2	81.2^{ab}	3.5
Quinacrine	70.0	6.0	3.88^{bc}	0.09	31.0^{bc}	1.6	83.8^{a}	2.0
Staurosporine	70.8	6.4	3.92^{bc}	0.08	33.0^{bc}	3.2	81.7^{ab}	4.1
Pb	70.0	5.5	3.83^{ab}	0.11	33.7^{b}	2.4	80.0^{ab}	1.8
Pb + Charybdotoxin	70.8	5.7	4.00^{c}	0.13	32.0^{bc}	2.0	76.0^{b}	2.1
Pb + Quinacrine	69.2	7.0	4.00^{c}	0.00	30.0^{bc}	2.0	79.7^{ab}	3.0
Pb + Staurosporine	68.3	5.1	3.92^{bc}	0.05	29.5^{c}	3.0	79.7^{ab}	3.3

4. Discussion.

The incubation of ram spermatozoa in medium containing 50 ng/ml of Pb^{2+} had negative effects on sperm viability and acrosome integrity, as we had observed in previous in vitro studies (Castellanos et al., 2008, 2013). Moreover, we found a higher quality of the movement of spermatozoa incubated with Pb^{2+} compared to controls. These changes may resemble those occurring during the spermatozoa capacitation related to the increase of membrane permeability and motility. In fact, some authors have found that these changes induced by Pb^{2+} could be in part inhibited with a chemical membrane effector like charybdotoxin that can act as a blocker of the capacitation process (Benoff et al., 2000).

However, in the present study all the tested inhibitors of capacitation also had a negative effect on acrosome integrity and then Pb^{2+} had none additional effect on the acrosome integrity. Specifically, acrosome integrity has not been additionally reduced by Pb^{2+} in samples incubated with charybdotoxin, quinacrine or staurosporine, as occurred with samples without these effectors. Similarly, samples incubated with these effectors showed none additional reduction in sperm viability when incubated with Pb^{2+}, compared with the samples incubated with effectors but without Pb^{2+}. However and more importantly, none of the potential blockers at the tested concentration had a protective effect against Pb effects on acrosome integrity or sperm viability. In fact, the lowest acrosome integrity was found in sperm treated with Pb^{2+} and staurosporine; and the lowest sperm viability was found in sperm treated with Pb^{2+} and charybdotoxin (Table 2). The last could be explained because Pb^{2+} at low concentration competes with Ca^{2+} in $VGK^{+}C$ channels and may destabilize the binding area for channels blockers (Doyle et. al. 1998), being able to cancel de inhibitory effect of charybdotoxin on VGCK. Moreover, these effects seem to be unrelated to any increase of the membrane permeability for Pb^{2+}, because Pb uptake was higher for sperm incubated with quinacrine than with staurosporine or charybdotoxin (*Fig*. 2).

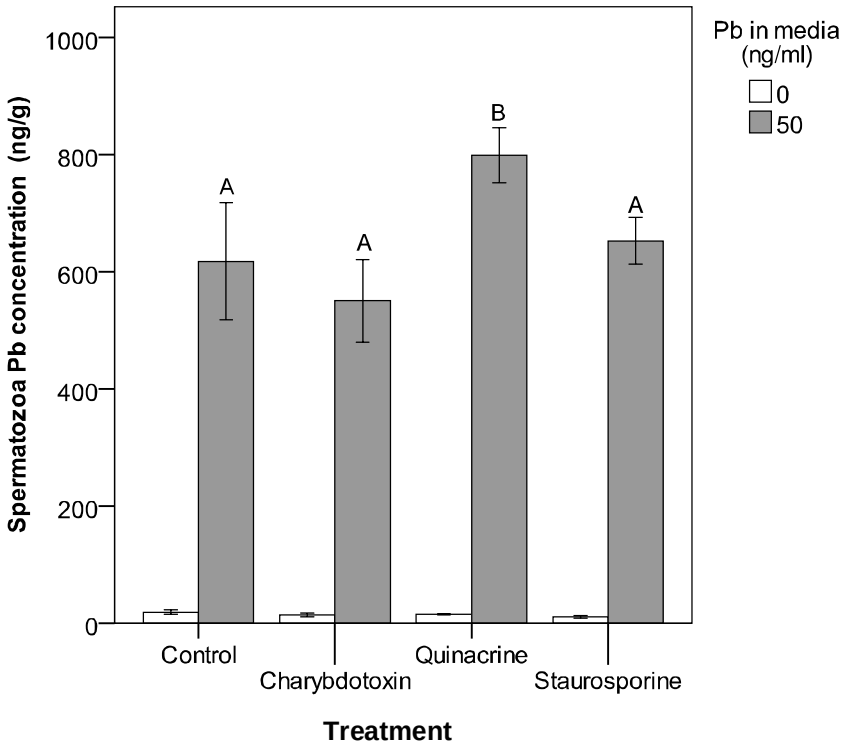


Fig. 2. Concentration of Pb (mean ± SE) incorporated into spermatozoa after incubation in media without or with Pb^{2+} and different potential inhibitors of capacitation. Different letters show differences between inhibitors in Pb treated samples. All the groups differed by Pb treatment.

As Pb^{2+} had a negative effect on sperm viability, the loss of acrosome integrity could respond to a more general failure of spermatozoa homeostasis related to the particular features of their membrane. The plasmalogens are the most abundant and exclusive glycerophospholipids that contribute on membrane stabilization and prevent the loss of the acrosome integrity by keeping the non-diffusible domains throughout its length (Paltauf, 1994; Gadella et al., 2008). These plasmalogens are more sensitive to oxidative reactions than their fatty acid ester analogues, due to the reactivity of their enolether function (Brosche and Platt, 1998). Moreover, spermatozoa membrane of ram, as well as in other animal species, have a high percentage of polyunsaturated fatty acids (PUFA) (Castellanos et al. 2008, 2010; Wolf et al., 1990), that makes it especially sensitive to lipid peroxidation. Reactive oxygen species (ROS) act at small amounts as

second messengers and can regulate the capacitation process (De Lamirande and O´Flaherty, 2008) and are produced during lysophosphatydilcoline-induced acrosome reaction (O'Flaherty et al., 2001). Similarly, boar sperm pre-exposed to a ROS-generating system had an increased acrosome reaction after incubation in capacitating conditions (Awda et al., 2009). Although ROS could be involved in these Pb-induced changes, we can not support the role of oxidative stress on the changes observed in our study. The concentration of Pb^{2+} used here (50 ng/ml) was tested before with ram spermatozoa to study effects on several oxidative stress biomarkers and the only significant finding was a decrease in lipid peroxidation that tended to disappear during the incubation respect to controls (Castellanos et al., 2008).

The capacitation process is characterized by the enhanced motility of the spermatozoa and the increase of membrane permeability followed by the acrosome reaction needed for the fertilization of oocyte. The hyperactive motility is a hallmark of sperm capacitation (Suarez, 2008) and is related with an increased protein-tyrosine phosphorilation and glycolysis in sperm tail (Arcelay et al., 2008). Moreover the initial loss of cholesterol has been suggested to affect the bilayer of the sperm plasma membrane making it finally more fusogenic (Gadella and Luna, 2014) and this is concomitant in the sperm head with the activation of protein kinases that also induce an increase of membrane fluidity. These membrane changes are also dependent on the presence of mM levels of extracellular Ca^{2+} and its transport into the cell (Carlson et al., 2007; Rejraji et al., 2009). Ca^{2+} is a divalent cation with which Pb^{2+} have similarities that can explain the disruption caused by this heavy metal on sperm quality parameters (Li et al., 2012). We have observed that the percentage of motile sperm was not affected by the incubation with Pb^{2+}, but the quality of their movement was significantly improved by the exposure to this metal in contrast to the results of other studies (Tvrda

et al., 2012). Moreover, sperm motility was also increased in samples incubated with Pb^{2+} and charydbotoxin or quinacrine (Table 2). The higher Pb^{2+} uptake, especially in samples incubated with quinacrine, may also support the hypothesis of some positive effect of Pb^{2+} on the progressive motility of spermatozoa at the incubation level of 50 ng/ml Pb^{2+} (*Fig. 2*). On the other hand, we expected that the blockage of $VGK^{+}C$ with charybdotoxin may have reduced the hyperpolarization of sperm membrane induced by Pb^{2+} and therefore the input of Ca^{2+} could be inhibited. On the contrary, we have seen that charybdotoxin could potentiate the effect of Pb^{2+} on the quality of spermatozoa movement. Therefore, the effect of Pb^{2+} on quality movement might be independent of the activation by Pb^{2+} of $VGK^{+}C$. An alternative mechanism may involve the action of HCO_3^- that activates sperm motility by increasing flagelar bat frequency through a pathway that requires adenylyl cyclase SACY Ca^{2+}-dependent (Carlson et al., 2007) (*Fig. 1*). Moreover, the cyclic nucleotides cAMP and GMP have also an important function in sperm motility and both are dependent on intracellular Ca^{2+} concentration (Aoki et al., 1999; Ren et al., 2001). In neurons exposed to Pb^{2+} the interaction between these cations has been also evidenced by the elevation of intracellular Ca^{2+} (Schanne et al., 1989). In this sense, the activation of PKC by Pb^{2+} may have induced the rise of intracellular Ca^{2+} (Schanne et al., 1997), causing the higher quality of sperm movement. In fact, the samples incubated with the PKC inhibitor staurosporine have shown the lowest quality of moment among the Pb-incubated sperm.

5. Conclusions.

The results of this in vitro study with low levels of Pb2+ (50 ng/ml) and inhibitors of membrane channels and enzymes involved in the capacitation process show that the toxic effect of Pb on sperm viability and acrosome integrity might not be exclusively produced by the interaction between Ca^{2+} and Pb^{2+} in membrane channels or in the activation of cytosolic enzymes. This can be concluded because none of the studied effectors had a protective effect against Pb^{2+} or even in the cases of staurosporine and charybdotoxin enhanced the effects of Pb^{2+}. The most significant result has been the higher uptake of Pb^{2+} in spermatozoa incubated with quinacrine compared to the controls and the spermatozoa incubated with staurosporine and charybdotoxin.

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Discusión General

1. Exposición al Pb en los ciervos de Sierra Madrona y Valle de Alcudia

El Pb es uno de los metales pesados con presencia más generalizada en el ambiente como resultado del uso que se ha hecho del mismo para muchas actividades humanas a lo largo de la historia. Esto ha llevado a poner en práctica importantes actividades de extracción minera de este metal, que en lo que se refiere a la provincia de Ciudad Real ha derivado en un registro de alrededor de 484 minas actualmente cerradas en un área de 2500 km^2 en la zona sur y suroeste de la provincia, y sobre las que no se han aplicado ningún tipo de medida de recuperación ambiental. Muchas de estas minas se encuentran dentro del recientemente creado Parque Natural del Valle de Alcudia-Sierra Madrona o su entorno. Como resultado de la explotación minera en esta zona ha habido un aporte antropogénico de metales al suelo, principalmente de Pb y Zn, al ser éstos los elementos mayoritarios de las venas metalíferas, y en menor medida de Ag, Cu, Sb, Sn y As (Palero-Fernández y Martínez-Izard, 2005). Esto convierte a este espacio natural en un enclave con importante contaminación residual de metales y metaloides, que no solo se localiza en el suelo de las zonas aledañas a la explotación en las que se llegan a encontrar niveles medios de Pb de 15.175 mg/kg en escombreras, sino que se ha dispersado registrándose concentraciones de Pb de 2.209 mg/kg en suelos circundantes y de 1.785 mg/kg en los sedimentos de los cursos de agua próximos (Higueras et al., 2011). Aunque el Pb se encuentra como especie inorgánica en forma de sulfatos, nitratos, óxidos o cloruros en principio poco solubles, como consecuencia

de la oxidación progresiva de los mismos ha ido pasando a la biota de esta zona. Así, en estudios anteriores realizados en estas áreas mineras, el Pb se encuentra acumulado en diferentes especies vegetales tales como la encina (*Quercus ilex*) y la jara pringosa (*Cistus ladanifer*), y en especial en las gramíneas se alcanzan valores medios de este metal cercanos a 100 μg/g peso seco (p.s.) (Reglero et al., 2008), para finalmente ser trasferido a ungulados silvestres que se alimentan en estas áreas como son el jabalí (*Sus scrofa*) y el ciervo común (*Cervus elaphus*). Este hecho lo prueba la presencia de mayor concentración de Pb y otros metales como el Cu, Cd, Se y Zn en diferentes tejidos de estos herbívoros en comparación con animales de zonas control no contaminadas (Reglero et al., 2008; Reglero et al., 2009a; Rodríguez et al., 2013). Aunque existe en la fauna de esta zona una exposición a múltiples elementos, sigue siendo el Pb el metal con mayor diferencia entre las zonas minera y control, tanto en el hígado (0.43μg/g *vs* 0.11 μg/g) como en el hueso (1.32μg/g *vs* 0.94 μg/g), lo que indica la existencia de una exposición crónica en los animales de estas áreas mineras.

Los efectos adversos que se pueden derivar de la exposición al Pb pueden perdurar, ya que de no ser eliminado fundamentalmente por la orina, por la bilis formando el completo Pb-glutatión, o por las heces (Alexander et al., 1986; Rabinowitz et al., 1980), es trasportado por la sangre y distribuido por el organismo. El Pb puede almacenarse temporalmente en tejidos blandos, fundamentalmente en el hígado, lo que lleva a encontrarlo aquí en concentraciones elevadas en los casos de exposición al mismo (Liu et al., 2012). También puede permanecer en los huesos y dentina desde donde se libera lentamente sobre todo del hueso trabecular o esponjoso y representa así una aportación endógena de este metal (Schütz et al., 1987a). Este último aspecto es importante en estudios anteriores en herbívoros de estas zonas mineras en los que se ha constatado un esfuerzo antioxidante con la disminución del α-tocoferol hepático en los

casos de mayor contenido de Pb en el hueso, lo que muestra el efecto a largo plazo de este metal en la defensa antioxidante de los animales expuestos a contaminación (Rodríguez-Estival et al., 2011).

Pese a que en los ciervos de áreas mineras, las concentraciones de Pb hepático han sido muy inferiores a las que se relacionan con signos clínicos de envenenamiento (30 μg/g p.s o 10 μg/g en p.f., Ma, 2011), los efectos tóxicos de este metal a nivel subletal se han evidenciado ya en estudios anteriores. Entre otros, se han observado una disminución en la mineralización del tejido óseo en los animales con mayor contenido hepático y óseo de este metal (Rodríguez- Estival et al., 2013), cambios en el sistema inmune en los casos de mayor concentración de Pb en el bazo (Rodríguez-Estival et al., 2013) o disminución en el hígado en un 16% de los ácidos grasos poliinsaturados (PUFAs n-3) unido a un descenso de hasta un 39% en los niveles de glutatión GSH) y de ésteres de retinol hepáticos (Reglero et al., 2009a; Rodríguez-Estival et al., 2011). Del mismo modo, este efecto se encontró en el testículo de ciervos machos adultos de zonas mineras en los que descendió el nivel de palmitato de retinol (Rodríguez-Estival et al., 2011). Estos últimos datos apuntan a que en esta zona de estudio la exposición a metales produce un efecto de estrés oxidativo por alteración en el balance entre prooxidantes-antioxidantes que puede estar detrás de los efectos observados (Rodriguez-Estival et al., 2013). Por último, también se vio que el ciervo de estas áreas parece ser una especie más sensible al daño oxidativo por la exposición a metales que el jabalí, ya que la modificación del status antioxidante del jabalí en estas áreas era menor que el que se producía en el ciervo (Rodríguez- Estival et al., 2011).

2. Efecto del Pb en la calidad espermática de los ciervos y en marcadores de estrés oxidativo

El efecto negativo del Pb bien de forma directa o mediado por estrés oxidativo sobre la calidad espermática es bien conocida. Así, la presencia de Pb en plasma seminal está asociada con oligospermia (poca concentración de espermatozoides) y astenospermia (poca motilidad de espermatozoides) tanto en hombres infértiles (Pant et al., 2003; Benoff et al., 2003a; Romamink et al., 2012) como en animales (Zheng and Li 2009; Lukacinova et al., 2012; Reglero et al, 2009b; Castellanos et al., 2010), y que además ha sido apoyada por estudios experimentales (Castellanos et al.2008; Selvaraju et al., 2011).

Ya en este estudio, en las muestras recogidas en 2005 (Reglero et al., 2009b, Capítulo 1), ha habido diferencias entre zonas minera y control en la concentración de Pb en los espermatozoides (2,97 *vs* 2,19 pg/10^6 cell) y en el testículo (37 ng/g p.s *vs* ND). En las muestras recogidas entre los años 2008 y 2010 se han encontrado valores más elevados de este metal y con mayores diferencias entre zonas, tanto en espermatozoides (30 *vs* 3 pg/10^6 cell) como en parénquima testicular (73 *vs* 58 ng/g p.s) y epididídimo (39 *vs* 27 ng/g p.s) (Castellanos et al., 2010, Capítulo 3).

Por otra parte, los niveles de Pb en hígado y hueso fueron significativamente superiores a los medidos en testículo, lo que indica que el tejido reproductor está más protegido frente a la entrada de este metal tóxico que los otros tejidos. Sin embargo, estos niveles de Pb detectados en testículo parecen ser suficientes para producir un estrés oxidativo y una disminución en los parámetros de calidad espermática (Reglero et al., 2009b; Castellanos et al., 2010). En cuanto a estos efectos, las diferencias entre

los ciervos de áreas mineras y control afectan a marcadores relativos a reproducción, como son el tamaño y peso de los testículos, viabilidad de membrana e integridad del acrosoma, niveles de glutatión total (GSH) y actividad de las enzimas antioxidantes glutation peroxidasa (GPX) y superóxido dismutasa (SOD), tanto en el testículo como en el espermatozoide. En algunos casos estas diferencias no han estado directamente asociadas a los niveles de Pb, sin embargo, la disminución en testículo en la actividad de la SOD ha estado asociada con un nivel de Cu inferior, mientras que la menor actividad de GPX en testículo se ha asociado con una mayor acumulación de selenio en este tejido, que podrían estar explicados por los requerimientos de estos elementos que tienen estas enzimas. De igual forma se encontró una relación positiva entre el porcentaje de viabilidad de la membrana del espermatozoide y las actividades de SOD y GPX en testículo. Estos resultados apoyan la idea de la importancia de la respuesta antioxidante para proteger la viabilidad y funcionalidad de la membrana frente a los ataques oxidativos (Fanaei H et al., 2014). Además, en los ciervos de áreas mineras unido a la disminución de los niveles de Cu en testículo se encuentra una mayor acumulación de Se y Zn en este tejido en relación a la detectada en la zona control. Estos datos apuntan a que la contaminación residual de las áreas mineras ha afectado a la homeostasis del Cu y Se y a su distribución por tejidos, ya que aunque los niveles de Cu son inferiores en testículo, son superiores en el hígado en relación a animales de la zona control (Reglero et al., 2009a).

Esta diferencia tan llamativa de distribución para el Cu entre zonas minera y control, que llegó a tener una concentración hasta un 43% inferior en testículo de animales expuestos, apareciendo sin embargo en mayor concentración en el hígado, podría explicarse en parte por una entrada especialmente activa de este metal en hepacitos ya que la proteína trasportadora de membrana es altamente específica (CTR1)

y está más presente en hepatocitos que en otros tipos celulares (Pena et al., 1999). Por otra parte, teniendo en cuenta que la excreción del Cu se lleva a cabo en un 98% a través de la bilis (Wijmenga and Klomp 2004), se podría explicar esta mayor presencia en hígado por ser el principal órgano de excreción.

Unida a la menor concentración de Cu observada en el testículo de ciervos de la zona minera se ha observado un mayor tamaño de los testículos que además está positivamente relacionado con los niveles de Se y Zn en testículo. Blottner et al. (1999) en ciervos expuestos al cadmio también observan un aumento del tamaño testicular que explican como un mecanismo compensatorio al daño en el epitelio germinativo de los corzo. Se sabe que la función de estos elementos en el mantenimiento de una buena calidad espermática y función reproductora (López-Rodríguez et al., 2013), ya sea interviniendo en la actividad de la GPX, como ocurre en el caso del Se; o bien por participar junto con las protaminas en los procesos de compactación de la cromatina en la cabeza del espermatozoide, como es el caso del Zn (Bianchi et al., 1992). Por otra parte, el aumento del epitelio germinal, permitiría compensar el descenso en la proliferación de las células espermatogénicas especialmente sensibles al ataque oxidativo (Falana et al., 2103). Además el aumento del epitelio germinativo con la mayor presencia de células de Sertoli compensaría la disminución de la Cu/Zn SOD, ya estas células son capaces tanto de sintetizar como de secretar esta enzima (Mrunk et al., 2002).

Por otra parte el hecho contradictorio de encontrar una relación inversa entre la actividad de GPX (que es una selenoenzima) y los niveles de Se en testículo puede tener varias explicaciones. Según estudios nutricionales, el Se es un oligoelemento esencial se requiere en pequeñas cantidades, de modo que un exceso del mismo puede bloquear la síntesis de la GPX por disminución en la expresión de mRNA (Gan et al.,

2002). Otro de los factores que han podido influir en este resultado es la forma química en que se encuentra el Se en el testículo de los ciervos, ya que aquí hemos determinado el Se total, pero también podría estar en forma orgánica en otras seleno-proteínas y no estar disponible para ser usado por la GPX. Se sabe que el 55% del Se en sangre se encuentra en forma orgánica unido a seleno-proteínas (Dumont et al., 2006). Por otra parte, estas seleno-proteínas pueden tener funciones adicionales a la de transportar Se. Las seleno-proteínas forman en los espermatozoides una estructura capsular en la pieza media imprescindible para la integridad del flagelo (Brown y Arthur, 2001). De este modo, la presencia de niveles elevados de Se podría deberse a su acumulación en seleno-proteínas diferentes de la GPX, reduciendo la disponibilidad para esta última. Para aclarar este resultado se está llevando a cabo la especiación del Se presente en estos testículos.

En las muestras recogidas entre los años 2008-2010 (Capítulo 3), se encuentra un descenso en la motilidad, en la viabilidad y especialmente en la integridad del acrosoma en los animales de la zona minera en correspondencia con una mayor concentración de Pb en el epidídimo y en los espermatozoides, sin embargo, se ha observado una mayor actividad de la GPX en el espermatozoide, que contrasta con la menor actividad encontrada en testículo en años anteriores (Reglero et al., 2009b). Este resultado podría explicarse porque en el epidídimo, donde el espermatozoide sufre importantes modificaciones hasta su maduración, la defensa contra el daño oxidativo corre también a cargo de una variante de la GPX que es Se-independiente llamada GPX-5, que es responsable de mantener la integridad de la célula durante este proceso (Chaborg et al., 2009). De este modo, la síntesis de esta enzima no seleno-dependiente podría aumentar como respuesta a la mayor presencia de Pb y daño oxidativo en el epidídimo para proteger mejor al espermatozoide en su fase post-testicular.

3. Pb, estrés oxidativo y daño en el ADN de los espermatozoides

Pese a la respuesta antioxidante de la GPX, otros antioxidantes en esperma como GSH y SOD que no han tenido diferencias significativas en relación a las zonas control (Capítulo 3). Esto puede explicar la disminución de todos los parámetros de calidad espermática en los ciervos de áreas mineras, además de encontrar alteraciones en los parámetros que miden daños en la molécula de ADN en el espermatozoide. Así, se ha encontrado un significativo aumento de 5,6% en el valor del índice de fragmentación de la cromatina (HDFI), que expresa daños por exposición a oxidantes (Martínez-Pastor 2009), así como un aumento de un 44% en el parámetro HDS relacionado con formas inmaduras, es decir con un menor grado de empaquetamiento del ADN alrededor de las protaminas. Este efecto puede venir condicionado por la mayor concentración de Pb y otros elementos originados por la contaminación minera en el parénquima testicular, epidídimo y espermatozoide en relación a la encontrada anteriormente. Las células espermiogénicas son menos capaces que otras de reparar los daños en el ADN producidos por agentes tóxicos y esto explica el efecto adverso del Pb en el proceso de espermatogénesis, incluso en concentraciones sin signos clínicos de envenenamiento (Ferrás et al., 2007; Nava-Hernández et al., 2009; Wang et al., 2013).

Por otra parte, el Pb altera seriamente el grado de empaquetamiento del ADN en la cromatina por unión con las protaminas por lugares específicos distintos a los que éstas tienen para unirse con el Zn (Quintanilla-Vega et al., 2000). Esta disminución en el grado del empaquetamiento producido por el Pb, hace a esta molécula más susceptible al ataque oxidativo tal y como se evidencia en otros estudios que muestran la relación entre el daño oxidativo en el ADN, fragmentación del mismo, alteraciones estructurales en la membrana del espermatozoide e infertilidad masculina con la

exposición al Pb (Erenpreiss et al., 2008; Taha et al, 2013). Estos datos están en consonancia con la alteración en los parámetros de calidad relacionados con la membrana que se han encontrado en esta tesis. Por otra parte, el significativo incremento en los valores de HDS en los espermatozoides epididimarios de las zonas mineras sería debido al incremento en el porcentaje final de formas inmaduras, que en otros estudios se ha relacionado con esterilidad idiopática asociada a una situación de estrés oxidativo por escasa defensa antioxidante en plasma seminal (Elshal et al., 2009).

4. Efecto del Pb en el perfil de ácidos grasos en testículo y espermatozoides

Otro aspecto a tener en cuenta para lograr una visión completa de los daños por estrés oxidativo en la función reproductora, es que el espermatozoide es una célula especialmente sensible al ataque de las EROs debido a que en su membrana hay una elevada cantidad de ácidos grasos insaturados (PUFAs). La presencia de varios doble enlaces en los PUFAs los hace especialmente vulnerable al ataque por especies reactivas del oxígeno (EROs) (Vernet et al., 2004; Whates et al., 2007).

Los ciervos de zonas mineras muestreados en el año 2004 han presentado diferencias en el perfil de ácidos grasos del espermatozoide y del testículo con respecto a los controles, que podrían ser consecuencia del estrés oxidativo causado por la contaminación minera y el Pb en especial. Así, los ciervos de la zona minera presentaban un mayor porcentaje de ácido linoleico (18:2n-6), ácido dihomo-γ-linoleico (20:3n-6), mirístico (14:0), el palmítico (16:0) y el menor porcentaje del ácido eicosatrienoico (20:3n-9) y el ácido araquidónico (20:4n-6) en testículo. Es precisamente el ácido araquidónico el que más baja en los espermatozoides (Castellanos et al. 2010). Estos cambios podrían ser debidos al descenso en la defensa antioxidante,

tanto en testículo como en espermatozoide, que podría haber afectado a PUFAs como el ácido araquidónico. Por otra parte, hay estudios que relacionan cambios en el perfil de los ácidos grasos con la disminución de calidad espermática y de fertilidad (Tavilani et al., 2007; Argov et al., 2007). Verstraeten et al. (2004) sugieren que el aumento de la fluidez de la membrana celular se relaciona con una disminución del ácido araquidónico y cambios apoptóticos relacionados con la distribución de la fosfatidilserina. En el caso de los espermatozoides, esta pérdida de fluidez puede ser responsable de la disminución de calidad espermática al disminuir la viabilidad de la membrana o alterar la integridad del acrosoma, tal y como se ha observado en los ciervos de áreas mineras (Reglero et al., 2009b; Capítulo 3). Por otro lado, el descenso del ácido araquidónico es especialmente relevante en la función espermática, ya que interviene en la síntesis de prostaglandinas que están involucradas en los procesos de capacitación del espermatozoide (Breitbart et al., 1995).

Por último habría que destacar que este descenso del 20:4n-6 observado en el espermatozoide y de forma menos marcada en el testículo por la exposición al Pb no coincide con el aumento de este ácido graso en tejidos como el hígado que muestran otros estudios (Mateo et al., 2003), lo que puede atribuirse a la compleja mezcla de metaloides a la que se ven expuestos los animales en estas áreas mineras. Así, se sabe que la presencia del Cd puede inhibir la expresión de algunas enzimas Δ-desaturasas que derivan en la síntesis final de ácido araquidónico (Kudo et al., 1996). Del mismo modo, la deficiencia en Cu afecta a la actividad de las estas enzimas (Whale et al., 1975), lo que lleva a pensar que la alteración en la homeostasis de estos metales por la presencia de Pb ha alterado los procesos de desaturación de los ácidos grasos de las membranas (Shingfield et al., 2008). Los resultados en el perfil de ácidos grasos en el testículo también se pueden explicar por la alteración de la actividad de estas enzimas,

en concreto por la disminución de actividad de las Δ-6 y Δ-5 desaturasas que afectaría a la conversión del 18:2n-6 a 18:3n-6 y después a 20:3n-6 (tras la acción de la enzima elongasa), de forma que se tendería a reducir la síntesis de ácido araquidónico con la consiguiente acumulación de sus precursores (Wallis et al., 2002).

Por último, los efectos del Pb el espermatozoide podrían no estar solo mediados por mecanismos relacionados con el estrés oxidativo. En Castellanos et al. (2008) (Capítulo 5) se observó que la exposición a diferentes concentraciones de Pb sobre una suspensión de espermatozoides de morueco (*Ovis aries*) provocaron efectos negativos sobre los parámetros de membrana, sin cambios en la actividad de la GPX, SOD ni en la concentración de los TBARS que indican peroxidación de las membranas. Estos mismos efectos directos del Pb sobre los parámetros de calidad sin peroxidación de membranas, se observan tanto *in vitro* (Huang et al., 2001) como *in vivo* (Xu et al., 2003) pero a concentraciones más elevadas que las utilizadas en este estudio (0,24 y 2,4 μM Pb^{2+}). Esto puede indicar que el espermatozoide de morueco es especialmente sensible al Pb y en parte puede deberse a que la proporción de estos PUFAs en su membrana llega a ser de un 45% del total (Castellanos et a., 2008).

La ausencia de peroxidación de lípidos de la membrana del espermatozoide in vitro podría explicarse en base a una cierta capacidad antioxidante de los plasmalógenos, unos glicerofosfolípidos especiales de estas células (Lessig y Fuchs, 2009), que son especialmente abundantes en los espermatozoides de morueco (Scott et al., 1967). Estos plasmalógenos presentan un enlace vinil-eter en el C_1 de la glicerina que puede actuar como primera diana de ataque de los radicales libres de modo que *in vitro* podrían en parte compensar la ausencia de otros antioxidantes que aparecen *in vivo* en el plasma seminal (Atiq et al., 2012).

5. Concentración tóxica del Pb en los espermatozoides

Por otra parte, en casos de exposición al Pb, los niveles de este metal en espermatozoides y plasma seminal son marcadamente más bajos que los encontrados en sangre ya que niveles de Pb en sangre de 93,1 ng/ml (9,31 µg/dl) corresponden a 2,02 ng/ml en plasma seminal y 0,047 ng/10^6 espermatozoides (Hernández-Ochoa et al., 2005). El descenso en la calidad espermática *in vivo* se encuentra con concentraciones mínimas de Pb de 0,07 y 0,24 ng/10^6 espermatozoides para afectar a la motilidad y viabilidad, respectivamente (Hernández-Ochoa et al., 2005). Por otra parte, en ciervos de áreas contaminadas del Valle de Alcudia con niveles de Pb en el espermatozoide de 3 pg/10^6 espermatozoides se ha encontrado una disminución en la calidad espermática unido a descenso en los niveles de antioxidantes y disminución de los niveles de ácido araquidónico (Reglero et al., 2009b; Castellanos et a., 2010). Efectos parecidos en la calidad espermática son los que se han encontrado en el ensayo *in vitro* de este estudio (Capítulo 4). Incorporando 50 ng/ml Pb^{2+} en el medio de incubación, la concentración de Pb detectada fue de 0,26 ng/10^6 espermatozoides, siendo la integridad del acrosoma el parámetro de calidad más afectado. Es por ello que estos niveles umbral de exposición al Pb encontrados *in vitro* pueden ser los apropiados para el estudio de los mecanismos de acción extracelular de este metal sobre el espermatozoide y sus resultados se podrían extrapolar a los efectos *in vivo* del Pb en los procesos post-testiculares. El hecho de ser la integridad del acrosoma uno de los parámetros de calidad espermática que más ha afectado la presencia de Pb, vuelve a evidenciar la sensibilidad de la estructura y funcionalidad de la membrana del espermatozoide frente al ataque de este metal y no siempre por mecanismos de estrés oxidativo, como se constatado en otros estudios *in vitro* (Benoff et al. 2003a).

6. Interacciones entre el Pb y procesos Ca-dependientes del espermatozoide

En el ensayo experimental del capítulo 6 se ha analizado cómo el Pb^{2+} aplicado en la concentración umbral medida anteriormente, puede disminuir la integridad del acrosoma por competencia, con el Ca^{2+} ya que el proceso de exocitosis acrosomal es, en gran parte, Ca-dependiente al ser este ion el que determina tanto la hiperpolarización de la membrana como la fosforilación de enzimas citosólicas que intervienen en el mismo (Demarco et al., 2003; Visconti et al., 2011). Se han utilizado tres sustancias potencialmente moduladoras del efecto del Pb, como son la charibdotoxina, que bloquea los canales de K-Ca dependientes (VGKC), la estaurosporina, que inhibe la actividad de la enzima proteinkinasa C (PKC), y la quinacrina, que actúa sobre la fosfolipasa A (PLA_2) (Benoff et al., 2000; Breitbart et al., 1995). No obstante, ninguno de los tres inhibidores ha sido efectivo para reducir los efectos del Pb sobre la viabilidad de la membrana o la integridad del acrosoma. Por el contrario, la incubación conjunta de espermatozoides de morueco con Pb y estaurosporina aumentó la pérdida de integridad del acrosoma, que podría explicarse porque la PKC actúa como diana celular para el Pb por interacción con Ca^{2+}, quedando activada con concentraciones nM de Pb (Sun et al., 1999), y que son los niveles usados en este experimento. Sin embargo, el hecho de encontrar una menor viabilidad de la membrana en el tratamiento de Pb con charidobtoxina hacer pensar que este parámetro de calidad espermática puede no estar directamente relacionado con cambios en la permeabilidad de la membrana por acción del Pb^{2+} en competencia con el Ca^{2+}, ya que la mayor entrada de Pb al interior del espermatozoide se produjo en el tratamiento con quinacrina. El aumento de la calidad de movimiento del espermatozoide en el tratamiento con quinacrina podría explicarse porque en este parámetro, está involucrado el ión bicarbonato HCO_3^- a través de una

ruta en la que participa la adenilciclasa, que es dependiente del Ca^{2+} en el citosol (Suarez et al., 2008). Por lo tanto, en esta ruta sí podría haber intervenido la interferencia por afinidad entre estos dos iones al ser en este tratamiento donde el Pb ha penetrado en mayor cantidad en la célula.

7. El plomo y la calidad espermática: una visión global de los posibles mecanismos de acción

El espacio natural del Valle de Alcudia y Sierra Madrona sufre una contaminación residual por minería que provoca en el ciervo común la acumulación de Pb en testículo y espermatozoides. Los parámetros de calidad espermática más afectados por esta contaminación han sido la viabilidad de la membrana y la integridad del acrosoma, que afectan a la reproducción de esta especie al comprometer ambas la capacidad fecundante del espermatozoide de los ciervos. El efecto del Pb sobre estos dos parámetros puede estar relacionado con un daño oxidativo, ya que se ha producido un descenso en la actividad antioxidante de la SOD y la GPX en testículo, y con alteraciones en la homeostasis de elementos como Cu y Se, que actúan como cofactores de dichos enzimas. En el caso de los ciervos de la zona minera, el descenso de la calidad espermática de los espermatozoides se ha presentado unida a una mayor actividad de la GPX en la célula, lo que puede indicar que existe un intento proteger al espermatozoide del daño oxidativo producido por la contaminación minera. Además, esta exposición a metales también ha provocado cambios en el perfil de ácidos grasos de espermatozoides y testículo de los ciervos, que podría tener también un origen en el estrés oxidativo inducido por el Pb al ser muchos PUFAs muy sensibles a la oxidación. De hecho, el cambio más notable ha sido la disminución del ácido araquidónico, descenso de especial

importancia para la capacidad fecundante del espermatozoide al tener este PUFA un importante papel en su proceso de capacitación. Por otra parte, la acumulación de Pb en los espermatozoides y epidídimo de los ciervos de áreas mineras se relaciona con un aumento en el índice de fragmentación del ADN, asociada también a una disminución de la calidad espermática.

Para conocer el efecto del Pb en etapas post-testiculares sobre la viabilidad espermática y la integridad del acrosoma del espermatozoide se ha reproducido este efecto *in vitro* utilizando para ello semen de morueco tratado con 50 ng/ml Pb^{2+}. Se ha constatado que la exposición *in vitro* al Pb de los espermatozoides produce una disminución de la calidad espermática al igual que ocurre *in vivo*, pero sin llegar a encontrar una relación significativa con biomarcadores de estrés oxidativo o con cambios significativos en la composición de ácidos grasos de la membrana espermática, tal y como se había visto en los estudios *in vivo*. Como mecanismo alternativo al estrés oxidativo, el experimento mediante el tratamiento *in vitro* de semen de morueco con charidobtoxina, estaurosporina o quinacrina que son sustancias moduladoras de funciones Ca-dependientes de los espermatozoides, no ha reducido el efecto del Pb sobre la disminución de la viabilidad de membrana o la integridad del acrosoma. Por el contrario, se ha visto que un inhibidor de la PLA como la quinacrina podría agravar el efecto de este metal al aumentar la entrada de Pb al interior del espermatozoide.

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Conclusiones

1. La contaminación residual por minería de en el Valle de Alcudia y Sierra Madrona provoca en el ciervo común la acumulación de Pb en testículo y espermatozoides.
2. La viabilidad de la membrana y la integridad del acrosoma, que comprometen ambas la capacidad fecundante del espermatozoide de los ciervos, han sido los parámetros más afectados por la contaminación minera.
3. La disminución de la viabilidad de la membrana y la integridad del acrosoma de los espermatozoides de ciervos ha estado relacionada con el descenso en la actividad antioxidante de la SOD y la GPX en testículo y con alteraciones en la homeostasis de elementos como Cu y Se, que actúan como cofactores de dichos enzimas.
4. Los ciervos de la zona minera con menor calidad espermática han presentado una mayor actividad de la GPX en espermatozoides, lo que puede indicar que existe un intento proteger al espermatozoide del daño oxidativo producido por la contaminación minera.
5. La exposición a la contaminación minera ha inducido cambios en el perfil de ácidos grasos de espermatozoides y testículo de los ciervos, siendo más notable la disminución del ácido araquidónico que tiene un importante papel en el proceso de capacitación del espermatozoide.
6. La acumulación de Pb en ciervos debido a la contaminación minera se relaciona con un aumento en el índice de fragmentación del ADN, que a su vez se asocia con disminución de la calidad espermática.
7. El efecto del Pb sobre la viabilidad espermática y la integridad del acrosoma del espermatozoide en etapas post-testiculares se ha reproducido *in vitro* con semen de morueco tratado con 50 ng/ml Pb^{2+}.
8. La exposición *in vitro* al Pb de los espermatozoides produce una disminución de la calidad espermática, pero sin relación con biomarcadores de estrés oxidativo o con cambios significativos en la composición de ácidos grasos de la membrana espermática.
9. El tratamiento *in vitro* de semen de morueco con moduladores de funciones Ca-dependientes de los espermatozoides, como charidobtoxina, estaurosporina o quinacrina, no han reducido el efecto del Pb en la viabilidad de membrana o la integridad del acrosoma, y por el contrario en el caso de la quinacrina lo puede llegar a agravar al aumentar la entrada de Pb al interior del espermatozoide.