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METHODS FOR IMPROVING BOAR AND STALLION SEMEN QUALITY

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"Methods for improving boar and stallion semen quality:

plant antioxidants supplementation from grape and green tea and semen photo-stimulation".

Illustration by the Author, Beatrice Gadani.

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ABSTRACT

METHODS FOR IMPROVING BOAR AND STALLION SEMEN QUALITY

Handling of semen is very important for a successful artificial insemination in both pig and horse farming. Liquid storage and cryopreservation are the techniques routinely utilized for male gamete storage. One of the main problems during liquid and frozen semen storage is the loss of fertilizing potential due to “cold-shock”, a variety of harmful cellular alterations mainly related to an excessive production of reactive oxygen species (ROS) and alterations in antioxidant defense systems. This phenomenon is called oxidative stress. It is known that ROS play an important role in the process of sperm maturation and appropriate levels are essential for sperm hyperactivation and capacitation. However, ROS over-production could be detrimental for sperm cells due to the presence of high proportions of unsaturated fatty acids in their cell membrane and scarce cytoplasm, which consequently results in an inadequate amount of antioxidant enzymes and low protection against ROS. So far, many studies have been performed to improve the efficiency of sperm liquid and cryopreservation techniques, in order to increase the percentage of viable cells after storage and to enhance their quality and fertilizing ability. Supplementation of sperm preparation with different antioxidants gave interesting and promising results and, during recent years, use of plant antioxidants has been gaining the attention of several research groups.

Moreover, an increase in the fertilising ability of boar sperm has been demonstrated after photo-stimulation.

On these bases, the objective of the present thesis was to assess whether:

- Resveratrol (RESV) or Epigallocatechin-3-gallate (EGCG) supplementation of thawing boar semen extender is effective in influencing sperm quality parameters and in vitro fertilization ability (IVF) (**Papers 1-2**);
- EGCG and green tea extract polyphenols (GT-PFs) improve stallion semen parameters during cooling at 4°C (**Paper 3**);
- Photo-stimulation improves frozen-thawed boar semen quality (**Paper 4**).

PLANT ANTIOXIDANTS SUPPLEMENTATION

Resveratrol (3,4',5-trihydroxy-trans-stilbene; RESV) is a polyphenolic natural product widely consumed in the Mediterranean diet being it present in peanuts, grapes and wine. RESV has been reported to possibly act as an antioxidant thanks to its ability to reduce mitochondria ROS production, scavenge superoxide radicals, inhibit lipid peroxidation, and regulate the expression of antioxidant cofactors and enzymes.

Epigallocatechin-3-gallate (EGCG) is the major polyphenol in green tea (*Camellia sinensis*) and is reported to possess a high level of antioxidant activity.

PAPER 1 – "Resveratrol and Epigallocatechin-3-gallate addition to thawed boar sperm improves in vitro fertilization."

The objective of this first paper was to assess the effect of different concentrations of RESV (0.5, 1, and 2 mM) or EGCG (25, 50 and 100 µM) added for 1 h at 37°C to thawing boar semen extender on sperm quality parameters (viability and acrosome integrity) and in vitro fertilization (IVF) outcome.

Both antioxidants, even if not able to exert any effect on sperm quality parameters (viability and acrosome integrity), efficiently improved in vitro penetration rate at all the concentrations tested. Moreover, the highest concentration of RESV (2mM) and the first two low doses of EGCG (25 and 50 µM) significantly increased the total efficiency of IVF.

PAPER 2 – "Combined effects of Resveratrol and Epigallocatechin-3-gallate on post thaw boar sperm and IVF parameters."

The aim of the second paper was to study the effect of supplementing boar sperm thawing medium with most powerful concentrations of EGCG (50 µM) and RESV (2 mM) tested in the first paper, alone or in combination, on in vitro fertilization (IVF) and to study the effect on boar sperm motility (assessed by CASA), viability, acrosome integrity, mitochondrial function, lipid peroxidation and DNA integrity (assessed by flow cytometry) and protein tyrosine phosphorylation (assessed by immunofluorescence). Thawed sperm cells were incubated 1 h at 37°C in BTS with or without the antioxidants; after incubation, an aliquot of sperm suspension was used for IVF trials, another was capacitated for 1 h at 39°C 5%CO_2 in Brackett and Oliphant's for immunolocalization of tyrosine-phosphorylated proteins, while another aliquot was used for sperm analysis. Finally, an aliquot of sperm suspensions was incubated for further 3 h in BTS at 37°C and, at the end of the incubation, flow cytometric sperm and CASA analysis were performed. The penetration rate was strongly

enhanced by RESV, alone or associated with EGCG; EGCG increased penetration rate as well but to a lower extent; tyrosine phosphorylated protein immunolocalization, used as capacitation parameter, was not affected by the different treatments.

Sperm motility was negatively affected by RESV (either alone or associated with EGCG) in comparison to control and EGCG groups both at 1 and 4 h; this effect was evident both in average motility parameters and in single cells kinematics (studied by cluster analysis). Viability, acrosome integrity, mitochondrial functionality and lipid peroxidation were not influenced by the addition of the antioxidants; finally, DNA integrity was negatively influenced by RESV (either alone or associated with EGCG) both at 1 and 4 h incubation.

Our findings demonstrate that the addition of RESV and EGCG positively affects in vitro penetration rate; anyway, some insights are needed to understand in particular if RESV (that showed the strongest effect) could be profitably used for artificial insemination in vivo, given the detrimental effect of this molecule on both sperm motility and DNA integrity.

PAPER 3 – "Epigallocatechin-3-gallate and green tea polyphenols do not improve stallion semen parameters during cooling at 4°C."

Stallion semen storage for artificial insemination is mainly based on liquid cooled storage. In many stallions this technique maintains sperm quality for an extended period of time (24–72 h) at 7°C. While this technique is commonly used in the horse industry, there can be a decline in fertility in some stallions, due to an inability of their sperm to tolerate the cool storage process.

The aim of the third work was to evaluate the effect of two natural antioxidants (EGCG at 20, 60 and 120 µM and green tea extract polyphenols at 0,001, 0,01 and 0,1 mg/ml) on some sperm parameters (sperm motility, viability/acrosome integrity and DNA quality) in extended semen immediately after its collection (T0) and after 2, 6, 24 and 48 h of cool storage. Two ejaculates from three trotter stallions were analyzed. No beneficial effect on the analyzed parameters was observed as the two antioxidants were not able to improve sperm quality during 48 h of storage. These results are in agreement with previous findings on the effect of different antioxidants reported by other researches, who have demonstrated that stallion semen keeps good antioxidant capacity after dilution for 24 h. In conclusion, the positive effect exerted by antioxidant molecules in other species is not confirmed in the equine one.

SEMEN PHOTO-STIMULATION

Photo-stimulation consists in the irradiation of cells with light in order to modify their metabolism. Previous works demonstrated that low laser irradiation could be useful to improve semen quality in avian, ram and bull spermatozoa. In boar liquid stored semen, the irradiation of spermatozoa improves, perhaps via a mechanism related to mitochondrial function, sperm motility and mitochondrial membrane potential, and increases the fertilizing ability of boar seminal doses.

PAPER 4 – "Photo-stimulation through LED prior to cryopreservation improves the cryotolerance of poor freezability boar ejaculates."

This last study sought to address whether photo-stimulation of boar sperm before cryopreservation could increase their cryotolerance and, thus, positively affect their function and survival. A main inconvenient of boar sperm cryopreservation, as it occurs in other species, is the individual variability of sperm cryotolerance which underlies the existence of good (GFE) and poor (PFE) freezability ejaculates. Therefore, the current study also evaluated whether the potential impact of photo-stimulation differs between GFE and PFE. Boar seminal doses were photo-stimulated before cryopreservation through MaxiPig® equipment which is a controlled-temperature device (set at 17°C) with triple LED that emits at a wavelength range of 620-630 nm and irradiates the samples for 30 min following a pattern of 10 min of light, 10 min of darkness and 10 min of further light. Sperm motility, viability, acrosome integrity and mitochondrial membrane potential were evaluated 30 and 180 min after semen thawing. LED photo-stimulation procedures increased the resilience of PFE to withstand cryopreservation, especially when viability, acrosome integrity and mitochondrial activity were evaluated at 180 minutes post-thaw. On the contrary, photo-stimulation had no effect on GFE and a detrimental effect on total sperm motility was observed in both types of ejaculates.

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Overall, these studies demonstrate that the addition of RESV or EGCG alone or in combination to thawed boar semen positively affect in vitro penetration rate while no positive effects were registered in cooled stallion semen up to 48 h storage; as a consequence, the addition of these substances in cooling medium for stallion sperm storage is not useful. Therefore, it is evident that the effects of antioxidants may vary depending on species as well as on the way semen is processed for preservation (cooled vs. frozen-thawed).

Moreover, photo-stimulation may be considered as a potential tool to increase the cryotolerance of poor freezability ejaculates but more researches on the mechanisms underlying the detrimental effects on sperm motility are required, as this may allow to understand whether such increase in cryotolerance has a significant impact upon the fertilizing ability of PFE.

RIASSUNTO

METODI PER MIGLIORARE LA QUALITÀ DEL SEME DI VERRO E DI STALLONE

La manipolazione del seme è un aspetto molto importante per un'inseminazione artificiale di successo sia nel settore suinicolo che nell'allevamento equino. Le tecniche di stoccaggio dei gameti maschili sono sostanzialmente due: la conservazione allo stato liquido a temperature di refrigerazione ed il congelamento in azoto liquido. Uno dei principali problemi durante la conservazione degli spermatozoi, sia allo stato liquido che crioconservati, è la perdita della capacità fecondante dovuta allo "shock da freddo" che causa una varietà di danni principalmente legati ad un'eccessiva produzione di ROS (specie reattive di ossigeno) e ad alterazioni dei sistemi di difesa antiossidanti. Questo fenomeno è chiamato stress ossidativo. È noto che i ROS svolgono un ruolo importante nel processo di maturazione degli spermatozoi e livelli adeguati sono essenziali per l'iperattivazione e la capacitazione. Tuttavia, la sovraproduzione di ROS può essere dannosa per le cellule spermatiche a causa della presenza di elevate quantità di acidi grassi polinsaturi nella membrana plasmatica e di scarso citoplasma che comporta una presenza di una quantità inadeguata di enzimi antiossidanti e quindi una bassa protezione contro i ROS. Finora sono stati condotti numerosi studi per migliorare l'efficacia delle tecniche di conservazione dei gameti maschili, al fine di aumentare la percentuale di cellule vitali dopo lo stoccaggio e di migliorare la loro qualità e la loro capacità di fertilizzazione. Negli ultimi anni la supplementazione di antiossidanti nei medium di conservazione degli spermatozoi ha dato risultati interessanti e promettenti e l'uso di antiossidanti di origine vegetale sta richiamando l'attenzione di diversi gruppi di ricerca.

Inoltre, recentemente è stato dimostrato che la foto-stimolazione del seme di maiale aumenta la farrowing rate ed il numero dei suinetti nati vivi mediante un meccanismo che potrebbe essere correlato alla funzionalità mitocondriale.

Su queste basi, l'obiettivo di questa tesi è stato quello di valutare se:

- la supplementazione singola o combinata dell'Epigallocatechina-3-gallato e del Resveratrolo nella fase di scongelamento del seme è efficace nell'influenzare la fecondazione in vitro (IVF) ed alcune caratteristiche qualitative del seme, quali vitalità, integrità acrosomiale, perossidazione lipidica, integrità del DNA e motilità (**Articoli 1-2**);
- l'aggiunta di Epigallocatechina-3-gallato e di polifenoli estratti dal tè verde migliora la qualità del seme di stallone durante la sua conservazione a 4°C (**Articolo 3**);

- la foto-stimolazione del seme, effettuata prima del congelamento, migliora la qualità del seme allo scongelamento (**Articolo 4**).

AGGIUNTA DI ANTIOSSIDANTI DI ORIGINE VEGETALE

Il Resveratrolo (3,4',5-triidrossi-trans-stilbene; RESV) è un polifenolo naturale largamente consumato nella dieta mediterranea in quanto presente in prodotti quali arachidi, buccia d'uva e vino. Il RESV possiede proprietà antiossidanti, grazie alla sua provata capacità di ridurre la produzione di ROS a livello mitocondriale, di inibire la perossidazione lipidica e di regolare l'espressione di cofattori ed enzimi antiossidanti.

L'epigallocatechina-3-gallato (EGCG) è invece una catechina particolarmente abbondante nel tè verde (*Camellia sinensis*) e nota per il suo alto potere antiossidante.

Articolo 1 – "Resveratrol and Epigallocatechin-3-gallate addition to thawed boar sperm improves in vitro fertilization."

L'obiettivo di questo primo articolo è stato quello di valutare l'effetto dell'aggiunta di diverse concentrazioni di RESV (0.5, 1, and 2 mM) o di EGCG (25, 50 and 100 µM), al medium di scongelamento del seme di maiale, su alcuni parametri qualitativi del materiale seminale (vitalità e integrità acrosomiale) e sull'efficienza della fecondazione in vitro (IVF). Entrambi gli antiossidanti utilizzati, pur non modificando alcun parametro di qualità spermatica (vitalità ed integrità acrosomiale), sono stati in grado di migliorare la percentuale degli oociti fecondati a tutte le concentrazioni testate. Inoltre, la concentrazione di RESV 2 mM e le due dosi (25 and 50 µM) di EGCG hanno aumentato significativamente l'efficienza totale della IVF.

Articolo 2 – "Combined effects of Resveratrol and Epigallocatechin-3-gallate on post thaw boar sperm and IVF parameters."

Lo scopo di questo secondo articolo è stato quello di studiare l'effetto dell'aggiunta combinata di EGCG (50 µM) e RESV (2 mM), alle dosi risultate essere più efficaci nel primo articolo, aggiunte al momento dello scongelamento del seme. Oltre all'efficienza della IVF sono stati valutati i seguenti parametri di qualità spermatica: motilità (valutata mediante sistema CASA), vitalità, integrità acrosomiale, funzionalità mitocondriale, perossidazione lipidica ed integrità del DNA (analizzati mediante citofluorimetria). L'immunolocalizzazione delle tirosine fosforilate è stata utilizzata come

parametro di capacitazione degli spermatozoi. I campioni di seme dopo scongelamento sono stati incubati per 1 ora a 37°C in presenza o in assenza degli antiossidanti. Dopo l'incubazione, una prima aliquota di seme è stata utilizzata per le prove di fecondazione in vitro, un'altra è stata capacitata per 1 ora a 39°C al 5% di CO2 in medium Brackett ed Oliphant, allo scopo di eseguire le prove di immunolocalizzazione ed, infine, una terza aliquota è stata utilizzata per le analisi di qualità spermatica. Questa terza aliquota è stata poi mantenuta in incubazione (sempre in BTS a 37°C) per altre 3 ore al termine delle quali (a 4 h di incubazione totale) sono state eseguite nuovamente queste ultime analisi con CASA e cito-fluorimetria.

La percentuale degli oociti fecondati è risultata significativamente più elevata grazie alla presenza del RESV, sia da solo che in associazione con l'EGCG. Quest'ultimo, allo stesso modo, ha ugualmente aumentato la percentuale di fertilizzazione ma con un effetto meno pronunciato. L'immunolocalizzazione delle tirosine fosforilate non è variata con nessun trattamento mentre la motilità spermatica in presenza di RESV (sia da solo che in associazione) ha subito un peggioramento, se comparata al gruppo di controllo o ai campioni trattati con EGCG, sia a 1 h che a 4 h di incubazione; questo effetto è risultato evidente sia nei parametri di motilità media che con una analisi delle caratteristiche di movimento degli spermatozoi. La vitalità, l'integrità acrosomiale, la funzionalità mitocondriale e la perossidazione lipidica non sono state influenzate dalla presenza degli antiossidanti; infine, il RESV (da solo od in associazione con EGCG) ha influenzato negativamente l'integrità del DNA sia dopo 1h che dopo 4 h di incubazione.

I nostri risultati dimostrano che sia il RESV che l'EGCG hanno effetti positivi sulla percentuale degli oociti fecondati; ulteriori studi devono essere condotti al fine di valutare il potenziale utilizzo del RESV (risultato essere il più efficace) per l'inseminazione artificiale, dati gli effetti negativi sui parametri di motilità ed integrità del DNA.

Articolo 3 – "Epigallocatechin-3-gallate and green tea polyphenols do not improve stallion semen parameters during cooling at 4°C."

La conservazione del seme di stallone per l'inseminazione artificiale è principalmente basata su un tipo di stoccaggio allo stato liquido e refrigerato. Per molti stalloni questa tecnica è in grado di supportare la qualità spermatica per 24-72 h a 7°C. Nonostante questa tecnica sia comunemente utilizzata nell'allevamento equino, gli eiaculati di alcuni stalloni possono presentare un

abbassamento della fertilità dovuto all'incapacità degli stessi di tollerare il processo di raffreddamento.

Lo scopo di questo terzo articolo è stato quello di valutare l'effetto di due antiossidanti naturali [EGCG 20, 60 e 120 µM; GT-PFs (green tea polyphenols) 0,001, 0,01 e 0,1 mg/ml], su alcuni parametri spermatici (motilità spermatica, vitalità/integrità acrosomiale ed integrità del DNA), quando addizionati al mestruo diluitore al momento del prelievo del seme (T0) e dopo 2, 6, 24 e 48 h dal raffreddamento dello stesso. A questo scopo sono stati utilizzati due eiaculati di tre diversi stalloni da trotto. Dall'analisi dei parametri sopracitati, non è stato osservato alcun effetto benefico in quanto entrambi gli antiossidanti non sono stati in grado di modificare la qualità spermatica nelle 48 h di stoccaggio del seme. I risultati ottenuti sono in accordo con studi precedenti, effettuati da altri autori che hanno dimostrato che Il seme di stallone mantiene una buona capacità antiossidante per circa 24 h dalla sua diluizione ad opera degli enzimi presenti nel plasma seminale, che non viene completamente eliminato durante tale operazione, e che l'aggiunta di un ulteriore supporto antiossidante non influisce sulla qualità spermatica. In conclusione, l'effetto positivo esercitato dalle molecole antiossidanti in altre specie non è confermata per la specie equina.

FOTO-STIMOLAZIONE DEL SEME

La foto-stimolazione consiste nell'irradiare le cellule con luce a diverse lunghezze d'onda allo scopo di modificare il loro metabolismo. Studi precedenti hanno dimostrato che irradiazioni laser a bassa frequenza possono essere efficaci nel migliorare la qualità del seme di ariete, toro e di varie specie aviari. Per quanto riguarda il seme di maiale conservato allo stato liquido, è stato dimostrato che la foto-stimolazione migliora, probabilmente con un meccanismo correlato alla funzionalità mitocondriale, la motilità spermatica ed il potenziale di membrana mitocondriale, ed è in grado di aumentare la *farrowing rate* e il numero dei suinetti nati vivi.

Articolo 4 – "Photo-stimulation through led prior to cryopreservation improves the cryotolerance of poor freezability boar ejaculates."

Quest'ultimo studio è stato messo in atto allo scopo di comprendere se la foto-stimolazione del seme di verro, prima di essere sottoposto al congelamento, è in grado di migliorarne la tollerabilità e quindi agire positivamente sulla sopravvivenza e sulla funzionalità spermatica nel post-scongelamento. Uno dei maggiori problemi riguardanti il congelamento del seme di maiale, come

accade anche in altre specie, è la variabilità individuale per quanto riguarda la capacità del seme di sopportare il congelamento, sottolineando l'esistenza di eiaculati con una buona (GFE) o scarsa (PFE) tollerabilità al congelamento. Quindi, il presente studio ha valutato se la foto-stimolazione ha effetti diversi tra i due gruppi, GFE e PFE. Dosi di seme di maiale sono state foto-stimolate prima del congelamento grazie ad uno strumento (MaxiPig®) in grado di controllare la temperatura (impostata a 17°C) e dotato di un triplo LED che emette in un intervallo di lunghezza d'onda pari a 620-630 nm e che ha irradiato i campioni alternando, ogni 10 min, luce e buio per un totale di 30 min. A 30 ed a 180 minuti dallo scongelamento del seme, sono stati valutati motilità, vitalità, integrità acrosomiale e potenziale di membrana mitocondriale. La foto-stimolazione a LED ha aumentato la resistenza dei PFE sottoposti al processo di congelamento, migliorandone la vitalità, l'integrità acrosomiale ed il potenziale di membrana mitocondriale, specialmente a 180 minuti dallo scongelamento. Al contrario, la foto-stimolazione non ha avuto alcun effetto sui GFE e sembrerebbe avere per entrambi i gruppi un effetto negativo sulla motilità.

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In conclusione, gli studi riportati in questa tesi dimostrano come l'aggiunta di RESV o EGCG, da soli od in associazione, al seme scongelato di verro induce effetti positivi sulla percentuale di oociti fecondati, mentre i polifenoli del tè non hanno alcun effetto benefico quando aggiunti al seme refrigerato di stallone; nello stallone, di conseguenza, l'utilizzo di tali sostanze al mestruo diluitore non è sfruttabile. Quindi, l'effetto degli antiossidanti può variare in funzione della specie animale presa in considerazione ed anche in base alla tecnica di conservazione del materiale seminale (refrigerazione o congelamento).

Inoltre, la foto-stimolazione potrebbe essere considerata come un potente strumento per incrementare la tollerabilità degli eiaculati di maiale con scarsa congelabilità. Tuttavia, si rendono necessari ulteriori studi per comprendere i meccanismi sottesi all'effetto negativo riscontrato sulla motilità spermatica; ciò permetterebbe di capire se questo aumento della tollerabilità al congelamento abbia poi un impatto significativo sulla capacità fecondante degli eiaculati foto-stimolati.

RESUMEN

MÉTODOS PARA MEJORAR LA CALIDAD ESPERMÁTICA DEL SEMEN DE CERDO Y CABALLO

La manipulación de semen es un aspecto muy importante para conseguir elevados rendimientos reproductivos tras la inseminación artificial, tanto en la ganadería de porcino como en la de equino. Las técnicas de preservación de los gametos masculinos son esencialmente dos: la conservación en estado líquido a temperaturas de refrigeración y la congelación en nitrógeno líquido. Uno de los principales problemas durante la preservación del esperma, tanto en la criopreservación como en la refrigeración, es la pérdida de la capacidad fecundante debido al "choque térmico por frío", que causa una variedad de daños principalmente relacionados con una producción excesiva de ROS (especies reactivas de oxígeno) y con alteraciones de los sistemas de defensa antioxidantes. Este fenómeno se llama estrés oxidativo. Se sabe que las ROS desempeñan un papel importante en el proceso de maduración de los espermatozoides y que niveles adecuados de ellas son esenciales para la hiperactivación y la capacitación espermática. Sin embargo, la sobreproducción de ROS puede ser perjudicial para las células espermáticas por la presencia de altas cantidades de ácidos grasos poliinsaturados en la membrana plasmática y por una cantidad insuficiente de enzimas antioxidantes debido al poco citoplasma presente, lo que implica una baja protección frente a dichas ROS. Hasta ahora, se han realizado numerosos estudios para mejorar la efectividad de las técnicas de conservación de los gametos masculinos, con el fin de aumentar el porcentaje de células viables después de la preservación, mejorar su calidad y su poder fecundante. En los últimos años, la suplementación con antioxidantes en los medios de conservación de los espermatozoides ha producido resultados interesantes y prometedores y el uso de antioxidantes de origen vegetal está atrayendo la atención de varios grupos de investigación.

Por otra parte, recientemente se ha demostrado que la foto-estimulación del semen porcino aumenta la tasa de parto y el número de lechones nacidos vivos, por medio de un mecanismo que, diversos autores, han relacionado con la actividad mitocondrial.

Sobre esta base, el objetivo de esta tesis fue evaluar si:

- la suplementación, sola o combinada, con Epigalocatequina-3-galato (EGCG) y Resveratrol (RESV) en la fase de descongelación del semen de cerdo es eficaz para aumentar el poder fecundante del semen *in vitro* (FIV) y en algunas características cualitativas del semen, como

la motilidad y viabilidad espermáticas, la integridad del acrosoma, la peroxidación lipídica y la integridad del ADN (**Artículos 1-2**);

- la adición de (EGCG) y polifenoles extraídos del té verde (GT-PFs) mejora la calidad del semen de caballo durante su refrigeración a 4°C (**Artículo 3**);
- la fotoestimulación del semen, realizada antes de la congelación, mejora la calidad del semen de cerdo descongelado (**Artículo 4**).

ADICIÓN DE ANTIOXIDANTES DE ORIGEN VEGETAL

El Resveratrol (3,4 ', 5-trihydroxy-trans-stilbene; RESV) es un polifenol natural ampliamente consumido en la dieta mediterránea, pues se encuentra en productos tan comunes como el maní, la piel de uva y el vino. El RESV tiene propiedades antioxidantes gracias a su comprobada capacidad para reducir la producción de ROS a nivel mitocondrial, inhibir la peroxidación lipídica y regular la expresión de cofactores y de enzimas antioxidantes.

El Epigalocatequina-3-galato (EGCG) es, en cambio, una catequina particularmente abundante en el té verde (*Camellia sinensis*) y es conocida por su alto poder antioxidante.

Artículo 1 – "Resveratrol and Epigallocatechin-3-gallate addition to thawed boar sperm improves in vitro fertilization."

El objetivo de este primer artículo fue evaluar el impacto de añadir diferentes concentraciones de RESV (0.5, 1 y 2 mM) o EGCG (25, 50 y 100 µM) al medio de descongelación del semen de cerdo. En concreto, se valoraron tanto parámetros de calidad espermática, como la viabilidad y la integridad acrosómica, como el poder fecundante in vitro (FIV). Ambos antioxidantes, si bien no modificaron ningún parámetro de calidad espermática (viabilidad e integridad acrosomica), fueron capaces de mejorar el porcentaje de oocitos fecundados en todas las concentraciones testadas. En efecto, la concentración de RESV 2 mM y de las dosis más bajas (25 y 50 µM) de EGCG aumentaron significativamente la eficiencia total de la FIV.

Artículo 2 – "Combined effects of Resveratrol and Epigallocatechin-3-gallate on post thaw boar sperm and IVF parameters."

El propósito de este segundo artículo fue estudiar el efecto de la acción combinada de EGCG (50 µM) y RESV (2 mM), a las concentraciones que dieron mejores resultados en el artículo anterior. En

este caso, ambos antioxidantes se añadieron en el momento de la descongelación del semen y, además de la eficiencia de la FIV, se evaluaron también los siguientes parámetros de calidad espermática: motilidad (evaluada por el sistema CASA), viabilidad, integridad acrosómica, función mitocondrial, peroxidación lipídica e integridad del ADN (analizados mediante citometría de flujo). La inmunolocalización de tirosinas fosforiladas se utilizó como prueba para la evaluación de la capacitación espermática. Después de la descongelación, las muestras de semen se incubaron durante 1 hora a 37°C en presencia o ausencia de antioxidantes. Transcurrida esta hora, se tomó una primera alícuota de semen para llevar a cabo pruebas de fecundación in vitro (FIV), otra fue utilizada para la capacitación en medio Brackett y Oliphant durante 1 hora a 39°C y CO_2 al 5%, con el fin de realizar pruebas de inmunolocalización y, finalmente, una tercera alícuota se utilizó para los análisis de calidad de los espermatozoides. Esta tercera alícuota se mantuvo en incubación (siempre en BTS a 37°C) durante 3 horas al final de las cuales (4 h de incubación total) se realizaron, de nuevo, los análisis con el sistema CASA y citofluorimetría.

El porcentaje de oocitos fecundados fue significativamente mayor en presencia de RESV, tanto solo como en asociación con EGCG. Este último, del mismo modo, incrementó también el porcentaje de oocitos fecundados in vitro, pero con un efecto menos marcado. La inmunolocalización de las tirosinas fosforiladas no varió con ningún tratamiento, mientras que la motilidad de los espermatozoides en presencia de RESV (solo o en combinación) fue significativamente inferior a la del control y a las muestras tratadas con EGCG, tanto después de incubar las muestras durante 1 o 4 h; este efecto fue evidente tanto en los parámetros globales de motilidad como en el análisis de las características de movimiento de cada espermatozoide. Por otra parte, aunque la viabilidad, la integridad acrosómica, la función mitocondrial y la peroxidación lipídica no se vieron influenciadas por la presencia de los antioxidantes, el RESV (solo o en asociación con EGCG) influyó negativamente en la integridad del ADN tanto después de 1 hora como después de 4 horas de incubación.

Con todo, estos resultados sugieren que tanto el RESV como el EGCG tienen efectos positivos sobre el poder fecundante del semen descongelado. Sin embargo, se deben realizar más estudios para evaluar los efectos de la adición de RESV (resultado más efectivo) en el rendimiento reproductivo del semen descongelado después de la inseminación artificial, y para determinar con más detalle la causa de los resultados adversos observados en la motilidad espermática y la integridad del ADN.

Artículo 3 – "Epigallocatechin-3-gallate and green tea polyphenols do not improve stallion semen parameters during cooling at 4°C."

En caballo, el semen preservado y destinado a inseminación artificial se suele conservar en estado líquido, concretamente refrigerado a 4-7°C. Esta técnica se utiliza comúnmente para la cría equina y, en muchos caballos, mantiene la calidad espermática durante 24-72 horas. Sin embargo, hay algunos caballos cuyos eyaculados presentan una elevada sensibilidad al choque por frío, lo que resulta en una disminución de la fertilidad del semen refrigerado. Por ello, el objetivo de este tercer artículo fue evaluar los efectos de dos antioxidantes naturales [EGCG 20, 60 y 120 μM; GT-PFs (polifenoles extraídos del té verde) 0,001, 0,01 y 0,1 mg/ml] sobre distintos parámetros de calidad espermática (motilidad, viabilidad, integridad acrosomica e integridad del ADN), después de su adición en el momento de la recogida de semen (T0) y después de conservar el semen refrigerado a 7°C durante 2, 6, 24 y 48 h. Para este propósito, se utilizaron dos eyaculados de tres caballos de trote diferentes. Después del análisis de los parámetros ya mencionados, no se observó ningún efecto positivo, porque la adición de ambos antioxidantes no mejoró la conservación del semen durante las 48 h. Los resultados obtenidos están de acuerdo con estudios anteriores, realizados por otros autores, que demostraron que el esperma de caballo mantiene una buena capacidad antioxidante durante, aproximadamente, las primeras 24 horas después de su dilución. Ello se debe a que los enzimas presentes en el plasma seminal no se eliminan por completo durante esta operación, de modo que un apoyo antioxidante adicional no afecta la calidad espermática. En conclusión, el efecto positivo de la adición de moléculas antioxidantes durante la preservación del semen en otras especies animales no se confirma para la especie equina.

FOTOESTIMULACIÓN DEL SEMEN

La fotoestimulación consiste en irradiar las células con luz de distintas longitudes de onda para modificar su metabolismo. Estudios previos han demostrado que la irradiación láser de baja frecuencia puede ser efectiva para mejorar la calidad del semen de humano, toro y varias especies de aves. Con respecto a al semen de cerdo preservado en estado líquido, se ha demostrado que la fotoestimulación mejora, probablemente a través de un mecanismo relacionado con la función mitocondrial, la motilidad espermática y el potencial de membrana mitocondrial, y es capaz de aumentar la tasa de partos y el número de lechones nacidos vivos.

Artículo 4 – "Photo-stimulation through led prior to cryopreservation improves the cryotolerance of poor freezability boar ejaculates."

Este último estudio se puso en marcha con el fin de determinar si la fotoestimulación del semen porcino antes de su criopreservación mejora su tolerancia a la criopreservación y si actúa positivamente sobre la supervivencia y la funcionalidad espermática en la post-descongelación. Uno de los mayores problemas relacionados con la congelación del semen de cerdo, como ocurre en otras especies, es la elevada variabilidad individual con respecto a la capacidad del semen para soportar la congelación, lo que determina la existencia de eyaculados con buena (GFE) y mala (PFE) congelabilidad. Por lo tanto, en el presente estudio se evaluó si la fotoestimulación tiene diferentes efectos entre los dos grupos, GFE y PFE. Las dosis de semen porcino fueron fotoestimuladas antes de la congelación mediante un equipo (MaxiPig®) con la temperatura controlada a 17°C constante y equipado con un triple LED que emite en un intervalo de longitud de onda igual a 620-630 nm. Dicho equipo irradió las muestras con un patrón alterno de 10 min de luz, 10 min de oscuridad y 10 min de luz. A los 30 y 180 minutos desde la descongelación del semen, se evaluaron la motilidad, la viabilidad, la integridad acrosómica y el potencial de membrana mitocondrial. La fotoestimulación mediante LED aumentó la resistencia de los PFE sometidos al proceso de congelación, mejorando sus parámetros de calidad espermática (viabilidad, integridad acrosómica y potencial de membrana mitocondrial), especialmente a los 180 minutos desde la descongelación. Por contra, la foto-estimulación no tuvo ningún efecto sobre los GFE y tanto en los GFE como en los PFE se observó un efecto negativo sobre la motilidad.

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En conclusión, los estudios descritos en esta tesis muestran que la adición de RESV o EGCG, solos o en combinación, en el semen porcino descongelado tiene efectos positivos sobre el porcentaje de oocitos fecundados. Por otra parte, los polifenoles del té no tienen ningún efecto positivo cuando son añadidos al semen de equino refrigerado, de modo que el uso de estas sustancias en el diluyente de refrigeración no es recomendable. Todo ello indica que el efecto de la adición de los antioxidantes durante la conservación del semen puede variar en función de las especies animales estudiadas y de la técnica de conservación del semen (refrigeración o congelación).

Además, esta tesis también demuestra que la fotoestimulación puede ser considerada como un instrumento útil para aumentar la criotolerancia de los eyaculados de cerdo con mala

congelabilidad. Sin embargo, se necesitan más estudios para comprender los mecanismos que subyacen los efectos de la fotoestimulación y es necesario también averiguar la razón de los efectos negativos observados en la motilidad espermática. Asimismo, estudios futuros tendrían que evaluar si la fotoestimulación de los eyaculados antes de la criopreservación tiene un impacto significativo sobre su capacidad fecundante después de la descongelación.

ABBREVIATIONS

FIRST LETTER	ABBREVIATION	NAME
symbols	↑	Increase/improve
	↓	Decrease/get worse
	↑↑	Increase more/improve more
	↓↓	Decrease more/get worse more
A	AA	Ascorbic acid
	AI	Artificial insemination
	ATP	Adenosine triphosphate
B	BHT	Butylated Hydroxytoluene
	BSA	Bovine Serum Albumin
	BTS	Beltsville Thawing Solution
C	Ca^{2+}	Calcium ion
	cAMP	Cyclic adenosine monophosphate
	CAT	Catalase
	CO_2	Carbon dioxide
	CPAs	Cryoprotective agents
	Cu_2^+	Cupric anion
D	DMSO	Dimethyl sulfoxide
	DNA	Deoxyribonucleic acid
E	EC	(-)-Epicatechin
	ECG	(-)-Epicatechin gallate
	EDTA	Ethylenediamine-tetra-acetic acid
	EGC	(-) Epigallocatechin
	EGCG	Epigallocatechin-3-gallate
	ETC	Electron transport chain
F	Fe_2^+	Ferrous ion
G	GSH	Glutathione reduced
	GSH-Px	Glutathione peroxidase
	GSH-Red	Glutathione reductase
	GSSG	Glutathione peroxide
	GT-PFs	Green tea polyphenols
	GTEs	Green tea extracts
H	h	Hour/hours
	H_2O_2	Hydrogen peroxide
	HCO_3^-	Bicarbonate anion or hydrogen carbonate
	He-Ne	Helium-Neon
	HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
I	ICSI	Intracytoplasmic sperm injection
	IVF	In vitro fertilization
	IVM	In vitro maturation
K	K^+	Potassium ion
L	L	Liter
	LPO	Lipid peroxidation/lipo-peroxidation

FIRST LETTER	ABBREVIATION	NAME
M	µg	Micrograms
	µM	Micro molar
	mg	Milligrams
	min	Minute/minutes
	mL	Milliliters
	mM	Milli molar
	mmp	Mitochondrial membrane potential
N	Na^+	Sodium ion
	NADH	Nicotinamide adenine dinucleotide
	NADPH	Nicotinamide adenine dinucleotide phosphate
	nM	Nano molar
	$^{\bullet}NO$	Nitric oxide
	NOS	Nitric oxide synthase
O	O_2	Oxygen
	$O_2^{-\bullet}$	Superoxide anion
	-OH	Hydroxyl group
	$OH^{\bullet}$	Hydroxyl radical
	$ONOO^-$	Peroxynitrite
	OS	Oxidative stress
P	PFs and PLow, PMed, PHigh	Polyphenols and Polyphenols at different concentration (Low, Medium, High)
	PKA	Protein kinase A
	pO_2	Oxygen partial pressure
	PTP	Protein tyrosine phosphorylation
	PTP	Protein tyrosine phosphorylation
	PUFA	Polyunsaturated fatty acids
	PUFA	Polyunsaturated fatty acids
R	RESV, Res, R	Resveratrol
	ROO•	Peroxyl radicals
	ROS	Reactive oxygen species
S	sAC	Soluble adenylate cyclase
	SIRT1	Sirtuin 1
	SOD	Superoxide dismutase
T	TRIS	2-Amino-2-(hydroxymethyl)propane-1,3-diol
U	UI	International Unit
	UV	Ultra-violet
W	w/v	Weight/Volume
	WTEs	White tea extracts

INTRODUCTION

SPERM STORAGE: AN OVERVIEW

In Veterinary Medicine, the history of research on artificial insemination (AI) is over two centuries old. So far, AI plays an important role in management and improvement of livestock productions. Its application has produced enormous benefits because not only is a simple, economical and successful technique, but also because AI has permitted genetic improvements, control of sexual diseases and reduction of lethal genes (Vishwanath, 2003). Only at the beginning of 2000s the world statistics for AI in cattle stand at 232 million doses of semen/year, in pig approximately 25 million/year and in horse, at today, the percentage of sport mares inseminated is very close to 90% in Germany and to 56% in France (Vishwanath, 2003; Roca *et al.*, 2006; Aurich, 2012). To made AI so accessible and useful around the world, a strong development in semen storage technologies was necessary to extend semen survival timeline (Bonadonna, 1980; Vishwanath, 2003).

It is well known that gamete cells, thanks to low temperatures, can reduce or stop their metabolic activities and this condition could be restored after re-warming (Mazur, 1984). This has made the preservation of semen possible, with great potential applications in agriculture, biotechnology, species conservation and clinical medicine (Yoshida, 2000).

In domestic animals, two are the possible kinds of semen storage: liquid or frozen state (Johnson *et al.*, 2000; Salamon and Maxwell, 2000; Vishwanath and Shannon, 2000). The first one is useful to preserve semen for few days, with temperatures higher than zero degrees. The second method is the most efficient for long-term sperm storage; it is also called “cryopreservation” and consists in a planned temperature reduction until -196°C (Holt, 2000).

From the first AI attempts in porcine specie, in the early 1900s, it was reported that sperm storage, both liquid and frozen, reduces survived cells, if compared to the original ejaculate. As temperature declines, the proportion of sperm cells with a normal structure is reduced (Johnson *et al.*, 2000).

Three are the main causes that influence sperm cell function after ejaculation and during storage: temperature (cold shock), dilution effect and length of storage (Johnson *et al.*, 2000):

- Cold shock occurs when freshly ejaculates are cooled quickly from body temperature to temperatures below 15°C (Johnson *et al.*, 2000) (*for a deep discussion on this topic see the second chapter “Cold shock and cryo-injuries” and the section dedicated to liquid storage in boar sperm cold shock of this thesis);*

- Dilution effect. It is well known that an excessive semen dilution leads to detrimental effect on ejaculate (Johnson *et al.*, 2000); at high dilution spermatozoa progressively loss their motility and membrane integrity, with a consequent decrease in cells viability (Garner *et al.*, 1997; Maxwell and Johnson, 1999; Johnson *et al.*, 2000). The so-called "dilution effect" occurs independently on the storage technique chosen (Garner *et al.*, 1997) and seems to be correlated with the reduction in protective components of seminal plasma (Maxwell and Johnson, 1999). However, the requirement of storage medium at an appropriate dilution is essential (*see the paragraph on boar and stallion extender medium components and their functions*).
- Finally, length and condition of storage are also important in the maintenance of sperm cell function as ageing of spermatozoa occurs, also when long-term media are used (Johnson *et al.*, 2000). For example, in liquid stored boar sperm, although DNA integrity is maintained, a decrease in viability and motility was reported, especially after 72-96 h (De Ambrogi *et al.*, 2006). Love *et al.*, 2002 demonstrated that sperm chromatin from sub-fertile stallions may decline already after 20 h of preservation. Regarding long-term cryopreserved human semen, no further loss in mean motility resulted until 36 months of storage in liquid nitrogen, if compared to the usually loss (approximately 50%) in motility values of the original ejaculate submitted to frozen-thawing process. However, if the initial semen quality is poor, a progressive decrease in motion parameters could be observed before this time (Smith and Steinberger, 1973).

In conclusion, ageing-related functional changes of stored spermatozoa, that could affect many structures of sperm cell (as mitochondria, flagellum, acrosome, plasma membrane), seem to be a physiological process not completely avoidable by preservation measures, even when additional cares are taken (as selection of semen with high quality) (Johnson *et al.*, 2000).

Sperm liquid storage

Boar

Liquid-stored semen is the most used during the ordinary practice at porcine AI centres. Indeed, more than 99% of all inseminations in the world are conducted with fresh ejaculates used the same day of collection or the following days.

Semen collected from boars is diluted in commercially available extenders and stored at 15-18°C. These extenders are used to create multiple insemination doses from a single ejaculate and are necessary to prolong sperm survival by providing energy to the cells, buffering the pH of the suspension and avoiding the growth of bacteria (Johnson *et al.*, 2000).

Many different boar semen extenders, either for short or long-term storage, are currently available. At present, the most common extenders used to dilute boar ejaculates are BTS (Beltsville Thawing Solution), initially used for thawing frozen semen (Pursel and Johnson, 1975), later adapted for liquid storage, and Zorlesco diluent, both for a storage until 5-days. In these diluents osmolarity is maintained by non-ionic compounds as glucose, that also create an intracellular acidosis, useful to increase cells survival. The loss of sperm motility is avoided through ions supplementation in media, as sodium-citrate or sodium-bicarbonate or potassium chloride (acting on Na^+/K^+ pumps, they can prevent K^+ intracellular exhaustion) (reviewed by Johnson *et al.*, 2000). Chelating substances for divalent metal ions (as EDTA – ethylenediamine-tetra-acetic acid) are also added to these extender formulations in order to reduce Ca^{2+} passage through plasma membrane, preventing a decrease in fertilizing ability due to premature sperm capacitation and acrosome reaction. Finally, during storage it is also important to reduce bacterial growth with antibiotics; therefore, penicillin or streptomycin are usually added to extenders (reviewed by Johnson *et al.*, 2000). When preservation exceeds 5 days of time, Androhep is usually the best extender for long-term boar sperm preservation. Androhep contains BSA (Bovine Serum Albumin) and HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) (Weitze, 1990). HEPES is a zwitterion organic buffer and is used to maintain a better sperm motility and to control pH value (Crabo *et al.*, 1972), that in boar has to be constant between 7.2-7.4 (Johnson *et al.*, 2000).

The main problem in boar semen storage is the spermatozoa extreme susceptibility to “cold shock”: a rapid cooling from 37°C (temperature at the moment of collection) to 15°C leads to a significant loss of motility (Johnson *et al.*, 2000). Compared to other species, plasma membrane of boar sperm is characterized by a higher ratio of unsaturated (PUFA)/saturated fatty acids and a lower percentage of cholesterol in phospholipidic bilayer (White, 1993). Low content in cholesterol results in a less cohesive and impermeable structure, making plasma membrane more susceptible to cold shock (Darin-Bennett and White, 1977), while high PUFA content makes sperm susceptible to lipids peroxidation (White, 1993). Therefore, a rapid cooling near to freezing point can induce plasma membrane changes that lead to a decrease in motility and metabolic activity, with damages in

acrosome and plasma membrane (White, 1993). In order that boar spermatozoa could acquire resilience to "cold shock", making possible their preservation in liquid state for days, without excessively compromising their quality, boar semen is usually stored at 15°C, reaching this temperature in several hours (Johnson *et al.*, 2000).

However, it is reported that farrowing rates, ranging from 65-70% when semen is used for AI in the first 2 days after its collection, decrease at about 50% with semen is hold for 5 days and litter size could also be affected by a long storage (Johnson *et al.*, 2000).

Stallion

In horse breeding, cooled semen is routinely used since about 30 years. During liquid storage, stallion spermatozoa can tolerate cooling to lesser temperature compared to porcine ones (Johnson *et al.*, 2000). Indeed, in equine species temperatures of about 5°C have been determined as optimal for maintenance of motility and fertility (Aurich, 2008).

During this process pH, osmolarity and bacterial growth are keep controlled, and a source of energy is usually added. Stallion extenders are generally based on egg yolk or milk. The sequestration of seminal plasma proteins, detrimental to semen quality, seems to represent a mechanism of sperm protection exerted by these two substances, avoiding cholesterol efflux from sperm membrane (important for capacitation and acrosome reaction) and, therefore, prolonging stored spermatozoa longevity (Pagl *et al.*, 2006). As milk and egg yolk include an enormous complex of biological substances, which may exert positive but also detrimental effect on semen quality, new diluents with a more defined and constant formula are still studied (Aurich, 2008). However, at today, the most popular and useful worldwide medium for cooled stallion semen is Kenney's extender, simply based on glucose, milk and antibiotics (Aurich, 2008), and modified Tyrode's medium, that better maintains motility after semen centrifugation; the latter is an extender for preserving semen in liquid state when horse seminal doses have to be transported (Padilla and Foote, 1991).

Unfortunately, in addition to the main causes already discussed (cold shock, dilution effect and length of storage), other factors have been reported to negatively affect the quality of cooled equine semen. First of all, stallions can be classified as "good" or "bad" cooler, according to the suitability of their ejaculates for cooled-storage. This depends on the initial quality of ejaculate, but also on composition of its seminal plasma and sperm plasma membranes (Aurich, 2008).

Despite of the better tolerance of horse ejaculates to low temperatures than pig ones, cooling is still a critical step, especially from 18 to 8°C, and also in this species may result in "cold shock", that could contribute to a reduction in semen fertility due to sperm injuries. As in boar, this detrimental effect on sperm cells can be reduced by reducing cooling rate (in stallion usually set at < 0.3°C/min). Storage at higher temperatures (15°C) can be performed but under aerobic condition and with a higher concentration of antibiotic compounds (Aurich, 2008).

Centrifugation is another step during semen storage technique that affects equine semen quality because it can induce lipid peroxidation of sperm plasma membranes. However, centrifugation is essential to reduce seminal plasma concentration and to dilute ejaculate with a suitable extender (Aurich, 2005). It is well known that high concentrations of seminal plasma in cooled-stored stallion semen are detrimental to motility and fertility, especially for stallions with poor tolerance to cooling and storage (Jasko *et al.*, 1991; Brinsko *et al.*, 2000)

Despite all the improvements obtained during last years in equine semen preservation, the longevity of stored stallion ejaculates is still limited, when compared to boars or ruminants (Aurich, 2005). Indeed, the fertility of cooled equine semen is usually maintained only for 24-48 h: after that time, pregnancy rate dramatically decreases (Aurich, 2008).

Sperm cryopreservation

Three methods can be used to preserve gamete cells for long-term: cryopreservation, vitrification and lyophiliziation (Barbas and Mascarenhas, 2009; Yeste, 2016).

Lyophilization (or freeze-drying) is a technique that does not require liquid nitrogen for storage (Choi *et al.*, 2011). Initially it was used for preserving viruses, bacteria, yeasts and fungi for easy storage and transportation (McLellan and Day, 1995). Recently, freeze-drying has also been applied to preserve spermatozoa from mammalian species (Wakayama and Yanagimachi, 1998; Kwon *et al.*, 2004; Choi *et al.*, 2011). However, spermatozoa that withstand to this technique are motionless and unable to fertilize either in vivo or in vitro (Liu *et al.*, 2004). As a consequence, lyophilized spermatozoa can be successfully used only in fertilization through intracytoplasmic sperm injection (ICSI) (Keskintepe and Eroglu, 2015).

Vitrification differs from conventional cryopreservation (or "slow freezing") in freezing/cooling rate. Vitrification consists in directly plunging samples into liquid nitrogen, avoiding injuries due to intracellular ice crystallization and removing the economic cost of expensive programmable freezing equipment. Despite the quite good results of this technique for preservation of several types of reproductive tissue or cells, the very low tolerance of spermatozoa to permeable cryoprotective agents (CPAs) – that are used in high amount to ensure intracellular glass formation – makes conventional vitrification inappropriate for mammalian spermatozoa cryopreservation. However, studies on new technologies of sperm vitrification, for example in CPAs absence, are still going on (Isachenko *et al.*, 2017).

Cryopreservation, is the most efficient method for a long-term semen preservation in mammalian species so far (Holt, 2000). Spermatozoa were the first cells successfully stored with this technique and the use of frozen-thawed semen for AI still plays a central role in domestic livestock production (Curry, 2007). Cryopreservation is also essential to improve the genetics of domestic animals, to preserve rare breeds and in international germplasm exchanges. In biotechnology it is useful to preserve endangered species, to improve bank resources for genetic management programs, to solve infertility problems or life threating diseases, and to conserve/produce transgenic animals for biomedical researches (reviewed in Holt, 1997; Barbas and Mascarenhas, 2009).

Cryopreservation (also called "slow freezing") is a long and delicate process. Protocols vary among species, extenders and time-temperature rates. However, it can always be divided into three phases: cooling, freezing and thawing. Cooling is used to reduce spermatozoa metabolic activities, while freezing completely stops them. Normal functions can be finally restarted by thawing (reported in Barbas and Mascarenhas, 2009).

Cooling is the first step of sperm cryopreservation. Temperature is decreased from normal one to +4°C, permitting some expansion of cell lifespan (Medeiros *et al.*, 2002). Mature spermatozoon, being the terminal cell of spermatogenesis, has peculiar characteristics that make it extremely vulnerable to insults. Cytoplasm is largely lost during spermatozoa development, as much of endoplasmic defense systems. Chromosomes are highly condensed in crystalline status and any protein transcription can occur, leading to DNA-repair enzymes deficiency and impedance in protein replacement. Membrane integrity is maintained by a very little endoplasmic reticulum or Golgi apparatus. Moreover, a negative correlation between head sperm size and cryo-stability is proved, so each species presents different cryo-sensibility to low temperatures (Medeiros *et al.*, 2002;

Barbas and Mascarenhas, 2009). As for the high sensibility of sperm cell, it is reported that in all domestic animals a fast cooling induces lethal stress, also called cold shock, proportional to cooling rate, temperature interval and temperature range. For these reasons, cooling is usually slow and conducted very carefully. Refrigeration rate is set at about 0.5-1°C/min. However, when a slow temperature rate is used, phase changes in membrane lipid bilayer occur (Watson, 2000), as described for liquid-stored semen.

To reduce cold shock on sperm cells, semen is diluted with extenders, generally after ejaculation with a dilution rate of 2-5 folds (Barbas and Mascarenhas, 2009). Sperm cryopreservation extenders usually include, as for liquid storage mediums, a buffer (such as TRIS), an energy supplier (sugars as glucose or lactose), salts (sodium citrate, citric acid) and antibiotics (penicillin, streptomycin) but also substances (CPAs) that can partially protect spermatozoa during freezing and thawing (Barbas and Mascarenhas, 2009).

Permeating CPAs (glycerol, dimethyl sulfoxide, ethylene glycol, propylene glycol) are commonly added to freezing extenders in order to increase membrane fluidity and to reduce cellular damages by intracellular ice formation. Glycerol is the universal cryoprotectant used for the cryopreservation of semen (Barbas and Mascarenhas, 2009). During freezing, as ice crystals are made exclusively of water, dissolved salts inside the cells increase, thus becoming detrimental for the spermatozoa. Glycerol, penetrating in sperm cells, can dilute the high concentration of salts (reviewed by Graham, 1996). Non-penetrating CPAs (egg yolk, milk or some sugars) are added in freezing extender formulas, and they can act only extracellularly. The exact mechanism of protection exerted by egg yolk is still relatively unknown (reported by Gibb and Aitken, 2016) but it clearly acts in maintaining plasma membrane integrity. Egg yolk lipoproteins could adhere to the cell membrane during freezing, thus restoring the lost phospholipids and, at the same time, inducing a transient change in phospholipids composition to prevent the rupture of the cell membrane and protect the cells (Alvarenga *et al.*, 2016). Sugars act on the osmotic pressure, dehydrating the cells and reducing the amount of intracellular water available for potential ice formation (Alvarenga *et al.*, 2016). However, spermatozoa are also sensitive to toxic effects caused by CPAs, resulting in osmotic shocks and chemical toxicity (reported by Watson, 2000). Slow freezing does not need high concentrations of CPAs as vitrification but, anyway, some compounds commonly used for other cells are unsuitable for sperm ones.

Cooling permits an expansion in sperm lifespan but, as refrigeration, can only limit, but not stop, sperm metabolic activities; therefore, aging process continues, leading cell to death (Medeiros *et al.*, 2002). To completely stop cellular metabolism, sperm have to be in frozen state.

Freezing is the second step of sperm cryopreservation. Usually sperm cells are frozen at -196°C, under liquid nitrogen (Barbas and Mascarenhas, 2009). This extremely low temperature is necessary for a long-term semen preservation: it is reported that cryopreservation at above -80°C is not stable, probably due to traces of unfrozen solution that still exist. At these low temperatures water only exists in crystalline or in glassy state and does not permit cellular diffusion; chemical reactions are stopped for insufficient thermal energy. The only reactions that occur at -196°C are photo-physical events, such as the formation of free radicals and the production of breaks in macromolecules, as a direct result of background ionizing radiation or cosmic rays. At these very low temperatures no enzymatic repairs can occur and these events can produce breaks or other deleterious DNA damages on cells, but only after a long-time (Mazur, 1984) and especially when the initial quality of ejaculate is poor (Smith and Steinberger, 1973).

Between -5°C and -15°C, but also at -30°C or below, even though ice begins to form in extender medium, the intracellular content remains unfrozen and super-cooled (Mazur, 1984). In this way water can flow from inside to outside the cell, and it freezes extracellularly. It is reported that both high and low cooling can lead to cell injuries. With rapid cooling, water does not flow completely outside the cell and forms cytoplasmic ice crystals, while slow cooling leads cells to an extremely dehydration state, resulting in hypertonic stress and lysis at thawing. Therefore, semen is generally frozen from +4°C to -140/-150°C at a rate of 0.5-100°C/min (Arav *et al.*, 2002), but the optimal rate is defined by each specific cell type and species, and it must to be enough low to avoid intracellular ice crystal formation and minimize cryo-injuries (Yeste, 2015).

Thawing phase is important for sperm survival in cryopreservation method because osmotic shock can kill cells, when they are returned to isotonic medium (Mazur, 1984). Generally, semen is thawed in water bath at 37-42°C for 20-30 sec in that, while freezing rate has to be slow, a fast thawing rate is required in order to avoid re-crystallization of any intracellular ice residues and cells are less exposed to high concentration of solutes and CPAs (Barbas and Mascarenhas, 2009).

Irrespective of the cryopreservation protocol performed, the extreme sensibility of sperm cells to all cryopreservation phases leads, at post-thaw, to a loss at about 50% of the initial motile

population in fresh ejaculate (Medeiros *et al.*, 2002). However, sensibility to cold shock differs among mammalian species. Among them, differences in sperm shape, volume, organelle size and composition exist: boar semen is the most sensitive, followed by bull, ram and stallion ones; dog and cat are somewhat sensitive, while rabbit and human are the less sensitive (Medeiros *et al.*, 2002).

Boar

In pig, semen cryopreservation technique is commercially available since 1975 (Johnson *et al.*, 2000) but only <1% of all worldwide AI are made using boar frozen-thawed doses (Rodríguez-Gil and Estrada, 2013; Knox, 2016). Compared to the use of the simply liquid-stored semen, cryopreservation in porcine species is still sub-optimal in terms of fertility and economic costs. Consequently, cryopreservation of boar semen has not been commonly used in commercial fields so far but it is useful for preservation of genetic resources, for improving the genetic progress and permitting the transportation of genetic material across countries (Johnson *et al.*, 2000).

Boar sperm cells differ in several aspects from those of other domestic animals, making these cells much more sensitive to low temperatures (Medeiros *et al.*, 2002). Immediately after its collection, boar ejaculate is extremely sensitive to cold shock and peroxidative damages, due to its plasma membrane rich in unsaturated phospholipids and low cholesterol (Yeste, 2015), and it is well known that cells become progressively less sensitive over the next few hours (Watson, 2000). In order to stabilize the lipid architecture of plasma membrane, usually an “holding time” of 10-24 h (during which semen can reach 15-17°C gradually), is set between sperm collection and the start of cryopreservation process (cooling) (Yeste, 2015). Rapid cooling from body temperature to temperatures near to the point of freezing leads to serious damages on membrane integrity and motility (Johnson *et al.*, 2000).

Consequently, in this species cooling has to be performed with controlled-rate temperature for a better quality at post-thaw, usually from 15-17°C to 4°C at 0.1°C/min (Yeste, 2015). However, there is a variation in sperm quality among individual boars, caused by male-to-male differences in the amount of long-chain PUFA in the sperm plasma membranes and, even if in a lesser extent, variations are also evident between different ejaculates from the same boar (Waterhouse *et al.*, 2006).

Many CPAs have been tested to improve pig semen cryopreservation but no one provides a better result in preserving boar semen than glycerol (Johnson *et al.*, 2000). Glycerol effectively improves post-thawed motility and viability of spermatozoa (Corcuera *et al.*, 2007; Gutiérrez-Pérez *et al.*, 2009) while other CPAs (as exythriol, xylitol, adonitol, acetamide and DMSO), even in relatively low concentration, can decrease acrosome integrity (Johnson *et al.*, 2000). However, also glycerol can have chemical and osmotic toxicities, depending on concentration (Buhr *et al.*, 2001). Once again, due to the extreme sensibility of boar sperm, the optimal level of glycerol (ranging 2-3%) necessary for an adequate cryoprotection has been reported to be detrimental for sperm perinuclear theca (Núñez *et al.*, 2013).

In order to improve boar cryopreservation outcome, many studies evaluated the effect of seminal plasma addition to freezing or thawing extender. In the first situation 5% of seminal plasma from good freezability ejaculates can improve motility and membrane integrity at post-thaw (Hernández *et al.*, 2007); in the second situation its supplementation has been reported to prevent capacitation-like-changes (Vadnais and Althouse, 2011). However, these effects could depend on extender and temperature of incubation (Vadnais *et al.*, 2005). Moreover, few information are present in literature upon the effect of seminal plasma supplementation on animal reproductive performance (Yeste, 2015).

Although cryopreservation in pig is still sub-optimal, studies to improve this technique continue to be performed by supplementing freezing and thawing media with different substances. In the last few decades most of the works published have focused on antioxidants supplementation, even of natural origin (*for a deep discussion on this topic see the four chapter "Antioxidants and sperm storage" and the section "Antioxidant supplementation of boar semen extenders: the most used in recent years" of this thesis).*

Stallion

In dairy cattle, bulls have been selected by the AI industry for many years on the basis of the ability of their sperm to withstand cryo-injuries, leading to an increase in uniform ejaculates with a good freezability (Loomis and Graham, 2008). Such selection based on reproductive performance has not been applied to stallions. They are commonly selected as sires basing on athletic performances, pedigree, and conformation (Varner *et al.*, 2008). As a result, there is a wide variation in semen freezability among individuals and the success of cryopreservation is lower than in other farm

animals (Loomis and Graham, 2008). Only 20% of stallions could be classified as “good freezers”, 60% are considered “fair/bad freezers” and the 20% of remaining stallions produce spermatozoa that do not withstand cryopreservation (Tischner, 1979). This individual stallion variation in the suitability of cryopreservation is much more important than, for example, differences among seasons (Aurich, 2016). Stallion semen is usually collected during the non-breeding season (autumn and winter), when the total means of motile and morphological intact spermatozoa in fresh semen are higher but, at the same time, in winter the decrease of cellular integrity by freezing/thawing is more pronounced. Hence, the post-thaw quality of frozen spermatozoa in ejaculates from breeding and non-breeding seasons are comparable (Blottner *et al.*, 2001).

As spermatozoa from individual stallions can respond differently to cryopreservation, it’s not hard to think why a standard protocol of cryopreservation does not exist. Consequently, the best choice is to optimize extender(s) and cooling/thawing rate for each valuable stallion (reviewed by Loomis and Graham, 2008). For example, the time required to achieve equilibrium at 5°C varies from 20 min to 2 h, according to the extender used (Alvarenga *et al.*, 2016). Usually, the freezing curve is performed in two steps: first, equine semen is cooled from room temperature to 5°C at a rate of 3-5°C/min, and then is frozen to -196°C at a rate of 20-50°C/min. Several protocols are available also for thawing but some studies indicate that 46°C for 20 sec or 37°C for 1 min are the most appropriate temperature and time rates (Alvarenga *et al.*, 2016).

A wide variety of commercial skimmed milk or casein based commercial extenders for horse semen exists. All of them provide the necessary nutrients for sperm metabolism, sperm function as buffers to control pH and osmolality, and protect the plasma membrane against cold shock and oxidative damage (Alvarenga *et al.*, 2016) but some ejaculates can be better cryopreserved using a specific extender than others (Graham, 1996). Many commercial semen freezing laboratories have now adopted a procedure, named “split-ejaculate test-freeze”, to evaluate and choice the best cryopreservation protocol resulted for new stallions. During this procedure, ejaculate (or ejaculates) collected from the stallion is divided and processed using different centrifugation techniques, extender compositions, cooling rates and packaging type, in order to verify whether this stallion can produce acceptable post-thaw quality with one (or more) of the procedures tested. However, from a commercial point of view, times and costs associated to the split-ejaculate test-freeze are an important drawback, both for stallion owners and freezing laboratories. Indeed, in order to select

the best protocol for each stallion, this test should be repeated three or four times on the same animal (Loomis and Graham, 2008).

Several techniques have been developed to increase the sperm quality of stallions as cushioned centrifugation for those stallions with low resistance to centrifugation or, when the initial quality of the equine ejaculate is poor, the use of density gradients, selecting only spermatozoa with progressive motility, cell integrity, and no morphologic defects, can be an option (Alvarenga *et al.*, 2016).

However, despite the fertility of frozen semen is acceptable when semen is of good quality, thanks to mares and stallions selection, and management practices are optimal (Loomis, 2001), in horse breeding the use of cooled semen is still preferred than frozen-thawed one, as in pig.

COLD SHOCK AND CRYOINJURIES

Cold shock is defined as a damage to cellular structures and functions, arising from a sudden reduction of temperature; this phenomenon regards different cell-types. The entity of injuries depends on the rate of cooling, temperature range and also cell-type (Watson and Morris, 1987).

In response to ambient temperature shift, organisms can change various physiological functions in order to survive. Cells may survive and respond to environmental stress with different strategies (Fujita, 1999; Ulusu and Tezcan, 2001; Feige *et al.*, 2013). They can adjust their unsaturated fatty acid amount in the plasma membrane according to the changes in the environmental temperature: if the temperature decreases, the amount of unsaturated fatty acids increases as a function of temperature and the membrane fluidity is modified in order to maintain its optimal function (Fujita, 1999). Cells also have an efficient DNA repair system in order to be protected from sources that can affect genome integrity, both endogenous and environmental. It is reported that multiple repair enzymes, as polymerases and kinases, are activated when DNA lesions occur, restoring its integrity (Sirbu and Cortez, 2013). Finally, a variety of proteins, mainly members of “heat shock proteins (HSPs)” family, can also be synthetized in response to environment adaptation, both in prokaryotes and eukaryotes. Functioning as molecular chaperones, they are the key components responsible for protein folding, assembly, translocation and degradation in many normal cellular processes and also under a wide variety of stresses, including exposure to UV light and temperature changes (Feige *et al.*, 2013; Park and Seo, 2015).

Mature spermatozoa are highly compartmentalized and transcriptionally quiescent cells. They are unable to regulate gene expression and, consequently, to synthesize new proteins or enzymes. This implies that sperm cannot modulate gene expression to face stressful environmental conditions and to regulate its physiology as other cells. Only post-translation protein modifications (as phospho/dephosphorylation), are known to play a significant role in some mechanisms regulating sperm function and response to environmental stress (Urner and Sakkas, 2003; Yeste *et al.*, 2014a).

Consequently, spermatozoa are extremely vulnerable to cooling, especially when temperature reaches the freezing point of water (first phase of cryopreservation) (Medeiros *et al.*, 2002). Lipid composition of membranes promotes the onset of cold shock more easily in sperm cells than in somatic ones (Drobnis *et al.*, 1993).

It is reported that a definite relationship exists between the ratios cholesterol : phospholipid and unsaturated : saturated phospholipids and the susceptibility of the spermatozoa to cold-shock (Darin-Bennett and White, 1977). Any plasma membrane is composed by both saturated and unsaturated phospholipids as well as a variable amount of sterols, such as cholesterol. Phospholipids are responsible for membrane fluidity, while cholesterol has a role in controlling this fluidity and providing a stable structure. At low temperatures, lipids undergo an alteration in physical state (Yeste, 2016), in particular in the vicinity of 5-15°C (Drobnis *et al.*, 1993). The first lipids to transit from fluid to gel state are unsaturated phospholipids in that they jellify earlier than others, with a restriction of the natural lateral movements of membrane phospholipids (Yeste, 2016). The effect of cooling on plasma membrane vary among species. As mentioned in the first chapter upon liquid storage, boar sperm plasma membrane is the most sensitive to cold shock in that it is characterized by a higher percentage of unsaturated phospholipids and a lower percentage of cholesterol in phospholipidic bilayer than other mammalian species. When lipids undergo an alteration in physical state and a lipid separation occurs, plasma membrane is destabilized. Molecules of cholesterol can be released and some integral membrane proteins, such as ion channels, are translocated and/or lose their function. Consequently, plasma membrane loses its selective permeability and an increase in ions influx, such as Ca^{2+} and HCO_3^-, from extracellular to intracellular space occurs. All these changes can lead to a phenomenon known as sperm capacitation-like changes (White, 1993; Yeste, 2016), also called cryo-capacitation because it is exacerbated by cryopreservation (Naresh and Atreja, 2015).

All the molecular changes occurring during sperm capacitation are not fully understood so far. However, it is well-known that capacitation is a sequence of physiological (such as sperm hyperactivation and acrosome reaction), and biochemical (cholesterol efflux, intracellular alkalinization, increase in intracellular concentrations of Ca^{2+} and HCO_3^-, cAMP, generation of reactive oxygen species and ATP) changes (Naresh and Atreja, 2015), which have been in part described above (about cold-shocked plasma membranes). Moreover, both in real-capacitation and in cryo-capacitation, all these biochemical alterations promote essential intracellular signaling events, such as protein tyrosine phosphorylation (PTP) (Naresh and Atreja, 2015). For these reasons, researchers speak about cryo-capacitation or capacitation-like-changes. This phenomenon should not be considered as a true capacitation, but rather as a by-passing of the capacitation process (Green and Watson, 2001). Indeed, when spermatozoa are cold-shocked, motility and metabolic

activity are irreversibly depressed and the acrosome and plasma membrane disrupted, with a consequently decrease in survival and fertilizing ability of stored mammalian spermatozoa (White, 1993; Yeste, 2016). This is true especially for cryopreserved semen: despite the use of the better cryopreservation protocol, the extreme sensibility of sperm cells to cold shock and osmotic stress leads to a loss at about 50% of the initial motile population in fresh ejaculate (Medeiros *et al.*, 2002).

OXIDATIVE STRESS AND SPERM CELLS

Oxidative stress (OS) is defined as an imbalance between the systemic production of reactive oxygen species (ROS) and the antioxidant defense system ability to detoxify the reactive intermediates or to repair the resulting damage (Agarwal *et al.*, 2014a).

ROS cover a large variety of oxidizing agents generated from the oxygen metabolism, such as superoxide anion ($O_2^{-\bullet}$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($OH^{\bullet}$). Due to the reside of "free radical", unpaired electron in the outer shell, they possess considerable reactivity with the nearest stable molecule and can attack a wide range of cellular targets, as carbohydrates, lipids, proteins and nucleic acids in order to obtain an electron (Aitken, 2017). This property is critical for defining ROS. Moreover, as the interconversion of these various reactive oxygen species and reactive nitrogen species is very rapid, other different and several redox entities might be involved in OS, as peroxynitrite ($ONOO^-$), derived by the reaction of $O_2^{-\bullet}$ with the biologically active nitric oxide ($^{\bullet}NO$), another free radical species (Aitken and Nixon, 2013).

Mammalian spermatozoa are professional generators of free radicals. It is well established that the presence of ROS is essential in sperm physiology because the capacitation process is considered itself as an oxidative event. While the central role of ROS in the induction of sperm capacitation is not in doubt, the molecular source of the free radicals and oxidants that stimulate capacitation are not well-known to date (Aitken and Nixon, 2013).

Three are the potential ROS sources inside of this gamete cell. The major one could be located in sperm mitochondria during cellular respiration by NADH oxidoreductase reactions in electron transport chain (ETC). The second might take place in plasma membrane by NADPH oxidases (NOX), and the last one involves L-amino acid oxidases, a cytosolic enzyme involved in the oxidative deamination of aromatic amino acids (Aitken *et al.*, 2015; Aitken, 2017).

L-amino acid oxidases is thought to be of a practical relevance only in the cryo-storage of spermatozoa, where a great number of dead sperm can release a large quantity of this enzyme. The direct contact with a wide concentration of free aromatic amino acids, derived from the presence of egg yolk in freezing extenders, can activate ROS production by L-amino acid oxidases, damaging the rest of live spermatozoa in ejaculate (Aitken *et al.*, 2015; Aitken, 2017).

Therefore, redox regulation of sperm capacitation seems to be driven by the other two ROS sources hypnotized and described above (NADH oxidoreductase reactions in ETC and NOX ones in plasma

membrane) that, at the end of the pathways (*see Figure 1 for a better explanation*), can mediate the changes well-known in sperm capacitation state (Aitken, 2017):

(1) the cholesterol oxidation and its efflux from plasma membrane (that allows a dramatically increase in plasma membrane fluidity to ensure, subsequently, sperm-oocyte fusion) (Cross, 1998);

(2) the up-regulation of protein tyrosine phosphorylation (one of the most important intracellular signaling events regulating sperm capacitation) (Naresh and Atreja, 2015);

(3) a typical sperm motility pattern called hyperactive (that provides spermatozoa with the necessary propulsion to penetrate the cumulus cells, a motion characterized by asymmetric flagellar movement with high amplitude and a significant displacement of the sperm head) (Ho and Suarez, 2001).

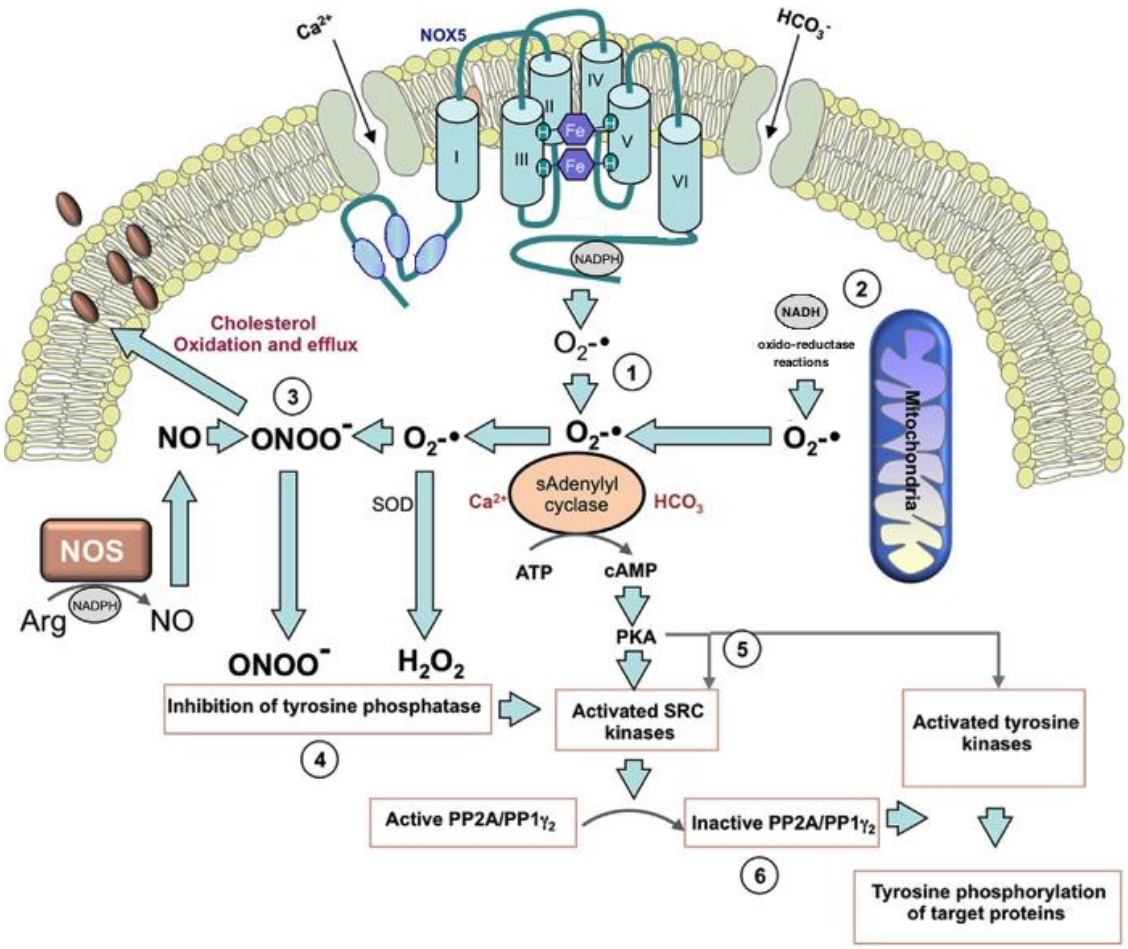


Figure 1 *- The proposed pathways mediating the redox regulation of sperm capacitation (Aitken, 2017, modified).*

*Mammalian spermatozoa generally possess NOX, transmembrane enzymes that are capable to produce $O_2^{-\bullet}$ transferring electrons from NADPH to molecular oxygen ($O_2^{-\bullet}$). This is one of the two pathways of ROS sources described above. This enzyme exists in different isoforms. Recently, one of these (NOX5) has been discovered in human sperm cells (Musset et al., 2012) and, being also a calcium-sensitive enzyme, could be effectively involved in sperm capacitation where calcium (Ca^{2+}) and bicarbonate ($HCO3^-$) ions play a central role in this process (**Point 1**). As already mentioned, the second source of $O_2^{-\bullet}$ in mammalian spermatozoa could be in the mitochondria, located in the midpiece, which generates a low level of ROS during steady-state respiration (**Point 2**). The $O_2^{-\bullet}$ could also combine itself with a •NO, a free radical derived by nitric oxide synthase action (NOS), resulting in $ONOO^-$. This powerful oxidant could mediate the cholesterol oxidation and its efflux from plasma membrane (**Point 3**) (reviewed by Aitken, 2017). Superoxide anion, derived by NOX and cellular respiration, is thought to participate in the activation of sAC/cAMP/PKA cascade (soluble adenylate cyclase, cyclic adenosine monophosphate, protein kinase A) (**Point 5**). PKA permits a significant increase in SRC kinases activity (a protein-tyrosine kinase family) that, in turns, can inhibit tyrosine phosphatase (**Point 6**) and, consequently, the dramatic up-regulation of tyrosine phosphorylation that characterizes capacitated spermatozoa. The combined action of reactive nitrogen species and H_2O_2 (generated by SOD activity – see "Antioxidants and sperm storage" chapter), concomitantly leads to the inhibition of tyrosine phosphatase activity, further enhancing protein tyrosine phosphorylation, as phosphate groups cannot be removed (**Point 4**) (Aitken, 2017). Finally, it is reported that $O_2^{-\bullet}$ could also support the initial regulation of hyperactivation (Lamirande and Gagnon, 1993).*

A low level of ROS exposure is physiologically required to drive the signal transduction processes associated with sperm capacitation, as already described. However, the production of free radicals is, at the same time, a double-edged sword for sperm. When over-exposure to such metabolites overcomes the sperm antioxidant defense systems (*described in "Antioxidants and sperm storage" chapter*) leads to OS that, in this case, is detrimental for sperm. Depending on the nature, amount, and duration of the ROS insult, these defects cause a significant damage to biomolecules (lipids, proteins, nucleic acids) for their free radical nature, leading to a decrease in fertilizing potential, as well as in the sperm capacity to support the embryonic development (Agarwal *et al.*, 2014a; Aitken, 2017). For example, the high amount of PUFA in plasma membrane leads to a more susceptibly of sperm cells to a cascade of chemical reactions called lipid peroxidation (LPO), caused by ROS presence. LPO is responsible for a decrease in membrane fluidity, an increase in non-specific permeability to ions, and an inactivation of membrane-bound receptors and enzymes (Agarwal *et al.*, 2014a). All these conditions are critical in fertilization event, as plasma membrane plays a central role in sperm-oocyte recognition and fusion. As plasma membrane, sperm DNA can be damaged by ROS. Usually, sperm DNA, being compacted and extremely condensed with protamines, provides protection from oxidative damage. However, in some cases, when poor compaction and incomplete

protamination of sperm chromatin exist, DNA is more vulnerable to OS, resulting in a production of base-free sites, deletions, mutations, and chromosomal rearrangements (Agarwal *et al.*, 2014a). As sperm cells only possess the first enzyme of DNA base excision repair system (named 8-oxoguanine glycosylase 1, responsible for releasing the oxidized base into the extracellular space), they have to carry their abasic sites into the oocyte for continuing the repair process. However, if DNA damage is extremely important or oocyte makes a mistake, the embryo development could be compromised (Aitken *et al.*, 2014).

Spermatozoa can protect themselves from OS damage by various antioxidants (e.g. glutathione, vitamin E, vitamin C) and antioxidant enzymes (as catalase, superoxide dismutase, and glutathione peroxidase or reductase) located in cytoplasm compartment, like other somatic cells (*described in "Antioxidants and sperm storage" chapter*), or in seminal plasma. However, usually the latter is in part removed during the normal laboratory procedures and the limited volume of sperm cytoplasmic space makes their defensive system weak for defying.

Moreover, reactive oxygen species can be produced not only at intracellular level (*as described above*), but also extracellularly by environmental factors. Under laboratory conditions two important factors can contribute to the accumulation of ROS, making their level dangerous to cell integrity. The first one is the cell exposure to an environment susceptible to OS. The oxygen partial pressure (pO_2) inside of incubators, the exposure to light and the use of culture media are just main examples (Agarwal *et al.*, 2014a). In assisted reproduction techniques (ART) laboratories, incubators usually exploit ambient air from the laboratory room. Consequently, pO_2 *in vitro* results higher than pO_2 recorded *in vivo*. In addition, at room temperature, pO_2 in the media is reported to be almost 20-fold higher compared to intracellular pO_2. This higher pO_2 activates various oxidase enzyme systems in the cells and contributes to increased ROS generation (Agarwal *et al.*, 2006).

Visible light induces photodynamic stress, leading to oxidative damage of unsaturated lipids and sterols within the membranes (Girotti, 1992), and can affects sperm motility and fertilization, depending on the wavelength employed, the amount of ROS produced (Zan-Bar *et al.*, 2005) and also on the duration of light exposure (light exposure of more than 5 min has been shown to cause a significant increase in the H_2O_2 levels) (Agarwal *et al.*, 2014a). UV and blue light generate high levels of ROS, resulting in a decrease in sperm motility and fertility (Zan-Bar *et al.*, 2005). Red light, contrarily, is reported to have positive effects on sperm cell (*see "Semen photo-stimulation" chapter*).

Culture media are designed to mimic the physiological environment in order to use them in ART treatments. Most of these media are supplemented, for example, with serum and/or albumin, where diverse oxidase activities have been found, while buffers used usually contain metallic ions (as Fe_2^+ and Cu_2^+), that can accelerate ROS production. Therefore, the medium itself could be a source of ROS during handling and culture (Martín-Romero *et al.*, 2008; Agarwal *et al.*, 2014a).

The second one is the sperm exposure to various *in vitro* manipulations (Agarwal *et al.*, 2014a). It is reported that ROS can be over-produced during sperm centrifugation. The duration of centrifugation seems more important than the force of centrifugation, causing more DNA fragmentation (reported by Agarwal *et al.*, 2014a). Cryopreservation is another handling process associated to OS occurrence (Tatone *et al.*, 2010). During cryopreservation, semen is exposed to a wide variety of cryoinjuries, caused by cold shock, osmotic stress, intracellular ice crystal formation, alteration in antioxidant defense systems, excessive ROS production and combinations of these conditions (Amidi *et al.*, 2016). It is no hard to think that all these factors contribute to sperm membrane damage and mitochondrial dysfunctions, inducing an OS status at intracellular level. Indeed, alterations to mitochondrial ETC and plasma membrane NADPH oxidase generate an increase in ROS production, that is reported not only during cooling/freezing phase, but also on semen thawing (reported by Tatone *et al.*, 2010). Moreover, the increase in DNA fragmentation reported after human sperm cryopreservation appears to be mediated by OS rather than caspases activation and apoptosis (Thomson *et al.*, 2009).

It should be noted that the effects of cryopreservation on ROS production in boar sperm are less clear than in other species (Yeste, 2016) because contrasting results are reported in literature. After cryopreservation, it is reported an increase in hydrogen peroxide, the major free radical in this species (Guthrie and Welch, 2006; Kim *et al.*, 2011). Other authors described that the basal ROS formation and membrane LPO are low, and comparable between fresh and frozen-thawed boar sperm (Guthrie and Welch, 2012). Despite this, supplementing sperm extenders media with antioxidants is positive for different species, including pig (Bathgate, 2011) (*see "Antioxidants and sperm storage" chapter*).

ANTIOXIDANTS AND SPERM STORAGE

Free radicals have to be constantly neutralized by agents that could break the oxidative chain reaction in order to maintain a ROS physiological level inside the cell. Normally, the presence of cellular antioxidants is helpful for this purpose but if cells undergo to oxidative stress (as during semen storage techniques) ROS are over-produced and endogenous antioxidants are not effective in preventing their detrimental effects on sperm cell (Bansal *et al.*, 2010). Spermatozoa are extremely vulnerable to oxidative stress because of their low content of scavenging enzyme in the cytoplasm and high concentration of membrane polyunsaturated lipids. Moreover, the abundance of mitochondria in the tail further complicates the resilience against free radical attacks (Amidi *et al.*, 2016).

Among all forms of sperm storage, freezing-thawing protocols are the ones that cause the greatest negative impact on sperm function and survival (Yeste, 2016). The main cryoinjuries in boar sperm are: reduction in motility, nucleoprotein alterations, DNA fragmentation (Flores *et al.*, 2008, 2011; Bathgate, 2011), mitochondrial activity reduction (de Lamirande *et al.*, 1997) and capacitation-like changes. This last alteration is not exactly equal to the physiological capacitation but results in a similar end-point to that observed in capacitated spermatozoa: changes in intracellular ion concentrations, alterations of lipid bilayer fluidity, modifications in motility pattern and increase in proteins tyrosine phosphorylation (Green and Watson, 2001; Bravo *et al.*, 2005).

One strategy to protect gametes from oxidative stress is the addition of antioxidant substances (Agarwal *et al.*, 2014b).

Antioxidants are scavengers that detoxify the excess of ROS (Agarwal *et al.*, 2012). There are two types of antioxidants: enzymatic and non-enzymatic (*Figure 2*).

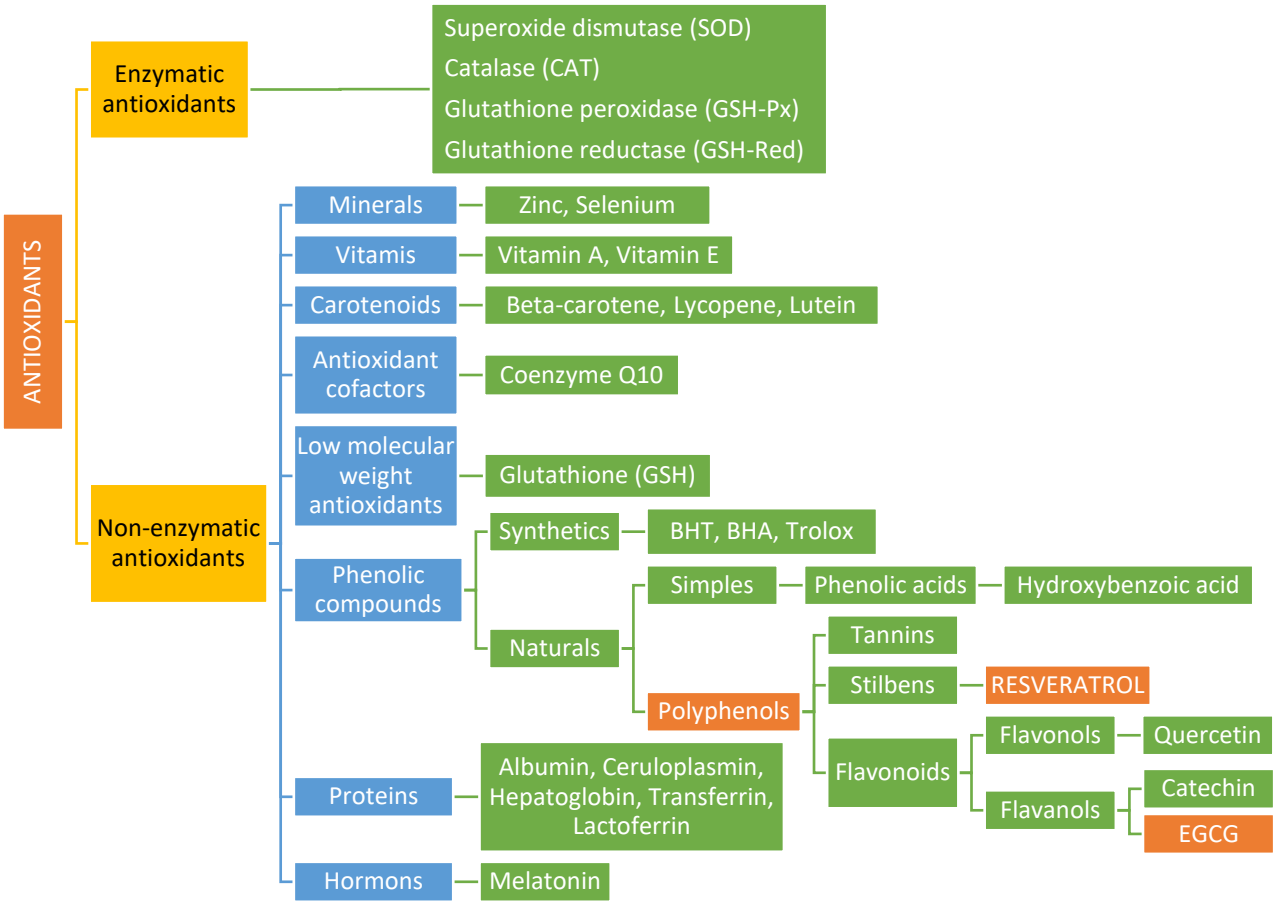


Figure 2 *- Schematic representation of enzymatic and non-enzymatic antioxidants (Bunaciu et al., 2015, modified).*

There are two main methods to improve the quality of stored semen using antioxidants. The first one is the antioxidants supplementation (usually non-enzymatic antioxidants) to the animal diet. Through intestinal absorption, antioxidants can reach the sex organs and subsequently increase the antioxidant levels in both seminal plasma and sperm (Bathgate, 2011). The second method is the antioxidant – enzymatic or not – addition directly to the semen extender medium (Bathgate, 2011). During cryopreservation, the supplementation could be in the cooling media, freezing or thawing media, or a combination of these (Bathgate, 2011), while in liquid storage preservation antioxidants are added to semen extender. However, their presence at high concentrations could have potential detrimental effects on sperm preservation, both in liquid and in frozen state (Yeste, 2016).

Enzymatic antioxidants are known also as "natural antioxidants" and commonly participate in sperm natural antioxidant defense system, neutralizing the excess of ROS and preventing their formation (Bansal *et al.*, 2010).

Superoxide dismutase (SOD) catalyzes dismutation of superoxide ($O_2^{-\bullet}$) into oxygen (O_2) and hydrogen peroxide (H_2O_2) (Cocchia *et al.*, 2011; Polimeni and Aperio, 2013). It scavenges both extracellular and intracellular superoxide anions and prevents lipid peroxidation of the plasma membrane (Perumal, 2014). Previous studies indicate that SOD supplementation to extender medium improves both motility and viability of equine chilled semen, while during cryopreservation it enhances in vivo fertilizing ability of frozen-thawed ram sperm (Silva *et al.*, 2011); Roca *et al.*, 2005 observed that the freezing extender supplemented with SOD could improve also the quality of frozen-thawed boar semen. Moreover, SOD appears to be a major antioxidant enzyme in boar seminal plasma, which could protect boar sperm from the harmful effects of ROS (Kowalowka *et al.*, 2008). In addition, Vallorani *et al.*, 2010 suggested that SOD, in association with seminal plasma, is effective in exerting some compensatory protection against the detrimental effects of storage on sorted semen. Recently, Zhang *et al.*, 2017 demonstrated that the addition of SOD to Modena extender improves boar sperm quality by reducing oxidative stress during liquid preservation at 17°C.

Catalase (CAT) is another enzymatic antioxidant that protects cells against H_2O_2 because it converts H_2O_2, generated by SOD, to water and oxygen (Polimeni and Aperio, 2013), as described below (*Figure 3*):

$$2(O^{-}{}_2{}^{\bullet}) + 2H^{+} \xrightarrow{SOD} H_2O_2 + O_2,$$

$$H_2O_2 \xrightarrow{Catalase} H_2O + \frac{1}{2}O_2.$$

Figure 3 *- Redox reactions catalized by SOD and Catalase (Bansal et al., 2010, modified).*

CAT addition to freezing extender is beneficial in post-thawed human, ram and canine semen, where this antioxidant induces a decrease of lipo-peroxidation and an improvement in sperm motility and viability (Michael *et al.*, 2007; Li *et al.*, 2010; Maia *et al.*, 2010). However, CAT cannot be useful to ameliorate semen quality in all species. For example, in chilled horse spermatozoa CAT has a detrimental effect on progressive motility (Aurich *et al.*, 1997) while in bull its supplementation exhibits different effects, depending on the type of extender used, both in cryopreservation and in

liquid storage (Foote, 1962; Asadpour *et al.*, 2011). In boar, Roca *et al.*, 2005 reported that CAT supplementation of freezing extender can positively act on in vitro embryo development.

Glutathione peroxidase (GSH-Px) and Glutathione reductase (GSH-Red) are other two important cellular enzymes with antioxidant proprieties (Agarwal et al., 2014 , Bansal, 2015). GSH-Px, a selenium-containing antioxidant enzyme with glutathione as the electron donor, removes peroxyl (ROO•) radicals from various peroxides including H_2O_2 and results in conversion of glutathione reduced (GSH) to glutathione peroxide (GSSG) in sperm (Bansal, 2015). GSH-Px and GSH-Red may directly act as antioxidant enzymes involved in the inhibition of sperm lipid peroxidation. GSH plays likely a role in sperm nucleus decondensation. Thus, in view of the great number of mitochondria in spermatozoa, these antioxidant mechanisms are important in the maintenance of sperm motility, rate of hyperactivation, and the ability of sperm to undergo acrosome reaction during sperm preparation techniques, especially in the absence of seminal plasma. A high GSH/GSSG ratio will help spermatozoa to combat oxidative stress. GSSG could be reduced back to GSH by the enzyme GSH-Red (Bansal *et al.*, 2010; Gadea *et al.*, 2011).

Non-enzymatic antioxidants, known as "synthetic antioxidants" or "dietary supplements", can influence, with a dietary intake, the antioxidant system of the entire body. Glutathione (GSH), minerals, vitamin E (α-tocopherol), vitamin C (ascorbic acid – AA), carotenoids, polyphenols, etc. can be supplemented to protect gamete cells from ROS during storage (Bansal *et al.*, 2010; Polimeni and Aperio, 2013; Agarwal *et al.*, 2014b).

Glutathione (l-γ-glutamyl-l-cysteinylglycine) is a tripeptide ubiquitously distributed in living cells; it is formed in the cytosol from glycine, cysteine, and glutamate (Agarwal *et al.*, 2014b). It plays an important role in the intracellular defence mechanism against oxidative stress. Glutathione peroxidase uses GSH to reduce hydrogen peroxide to H_2O and lipoperoxides to alkyl alcohols; the resulting oxidized glutathione (GSSG) is reduced to GSH by glutathione reductase using NADPH as the co-factor (Gadea *et al.*, 2011). In human spermatozoa a decrease in GSH content after cryopreservation has been reported and its addition (1 or 5 mM) to the thawing extender has been shown to increase the percentage of motile and progressively motile spermatozoa (Gadea *et al.*, 2011). It was demonstrated that GSH added to bovine semen extender results in a decrease of LPO level during freezing and in an improvement of fertility rates (Perumal *et al.*, 2011). Tris-citric acid extender supplemented with GSH improved the freezability of buffalo (Bubalus bubalis) bull spermatozoa in a dose dependent manner. Moreover, the addition of 2.0 mM GSH to the extender

enhanced the in vivo fertility of buffalo and bull spermatozoa (Ansari *et al.*, 2012), while in frozen goat semen GSH addition decreased acrosomal abnormality and supported post-thaw motility (Sinha *et al.*, 1996). In boar, the effects of GSH supplementation have been widely studied. In this species, GSH can improve fertilizing ability of frozen-thawed semen, both in vivo and in vitro, and can ameliorate the cryotolerance of ejaculates (*for details see Table 1*).

Precursors of GSH, as L-Cysteine, have also been successfully employed in mammalian sperm cryopreservation. Cysteine is a sulfur-containing amino acid and an important structural and functional component of proteins and enzymes. Cysteine has been shown to improve motility and morphology of post-thawed bull (Bilodeau *et al.*, 2001), ram (Uysal and Bucak, 2007) and goat (Bucak and Uysal, 2008; Memon *et al.*, 2012) sperm, and to maintain viability, chromatin structure and membrane integrity of boar sperm during liquid storage at 15°C (Szczesniak-Fabianczyk *et al.*, 2003). When L-Cysteine is added in extender medium during cryopreservation of bull and cat sperm, it results in a decrease in DNA damages and in a higher motility, compared to control groups (Thuwanut *et al.*, 2008; Tuncer *et al.*, 2010). In frozen boar semen, Cysteine can support viability and acrosome integrity (*for details see Table 1*).

The antioxidant action of Vitamin C or ascorbic acid (AA) consists in stopping the propagation of peroxidative reactions and in helping the recycling of vitamin E and glutathione oxidized (Polimeni and Aperio, 2013). The addition of ascorbic acid (4.5 mg/ml) to cryoprotective medium increased bovine sperm motility, movement characteristics and the integrity of acrosome and membrane (Hu *et al.*, 2010), while in infertile men reduces sperm DNA damages (Branco *et al.*, 2010). Moreover, AA, alone or in combination with GSH, has been reported to improve sperm cryotolerance in boar sperm (*for details see Table 1*).

Vitamin E or α-tocopherol plays a major role in antioxidant activities in that it reacts with lipid radicals produced during lipid peroxidation. This reaction produces oxidized tocopheryl radicals which can then be reduced by ubiquinone or by ascorbic acid (Bansal *et al.*, 2010). Vitamin E significantly improves post-thaw quality of cryopreserved human sperm, in terms of motility and DNA integrity (Kalthur *et al.*, 2011). In cat epididymal spermatozoa, Vitamin E supplementation has been reported to significantly improve post-thaw total motility, progressive motility, and membrane integrity (Thuwanut *et al.*, 2008). In porcine species, Vitamin E efficiently protects spermatozoa from cryoinjuries, reducing lipo-peroxidation and DNA fragmentations *(for details see Table 1).*

Other antioxidants

During the last years, the interest in the antioxidant properties of plant derived substances on animal and human semen quality grew up. Polyphenols represent a group of chemical substances that are common in plants. These compounds have been widely tested as additional substances on semen extenders, not only because they have been demonstrated to be effective scavengers of ROS (Higdon and Frei, 2003), but also because they have been found to exhibit higher antioxidant activity than synthetic antioxidants (Zhong and Zhou, 2013). This group includes molecules isolated in common dietary constituents as green tea (Khan and Mukhtar, 2007), with the presence of Epigallocatechin-3-gallate (EGCG) (Khan and Mukhtar, 2007; Chacko *et al.*, 2010), or Resveratrol (RESV) found in the seed and skin of grapes (Siemann and Creasy, 1992; Fernández-Mar *et al.*, 2012). As all polyphenolic compounds, EGCG and RESV have species-specific and opposite effects, according to the concentration of the molecule used (Murakami, 2014). Being their effects on boar sperm cells studied in this thesis, they will be well described in specific chapters (*see "Green tea polyphenols (GT-PFs) and Epigallocatechin-3-gallate (EGCG)" and "Resveratrol (RESV)" chapters*).

Antioxidant supplementation of boar semen extenders: the most used in recent years

Supplementation with various antioxidants improves both viability and motility of liquid or cyro-preserved boar spermatozoa. Boar sperm not only have a poor innate resistance to ROS, as all male gamete cells, but its plasma membrane is much richer in polyunsaturated fatty acids (PUFA) than that of other species. This can lead to high levels of lipid peroxidation because PUFA are easily hackable by ROS. Thus, the reinforcement of these resistances through addition of antioxidants can show a better effect than in other species (Peña *et al.*, 2004; Bathgate, 2011; Amidi *et al.*, 2016).

Table 1 *- Effects of antioxidant addition during boar sperm storage (liquid or frozen)*

Antioxidant	Point of antioxidant addition	Type of semen storage	Antioxidant concentration	Principal effects of antioxidant	Reference
BHT (vitamin E analogue)	Freezing	Frozen	from 0.2 to 0.8 mM	Improves the survival of spermatozoa after thawing	(Roca *et al.*, 2004)
			0.4 mM	Improves embryo development	
BHT	Freezing	Frozen	1 mM	Improves the fertilizing ability of post-thaw spermatozoa	(Trzcińska *et al.*, 2015)
CAT	Freezing	Frozen	200-400 UI/mL	Increases post-thaw viable sperm and in vitro embryo development	(Roca *et al.*, 2005)
Fennel (*Foeniculum vulgare*)	Freezing	Frozen	10 g/100ml	Improves sperm motility and viability; reduces lipoxidation	(Malo *et al.*, 2012)
GSH and AA	Freezing and thawing	Frozen	5 mM GSH + 100 µM AA	Improve boar sperm cryotolerance (sperm viability, motility, acrosome integrity and nucleoprotein structure)	(Giaretta *et al.*, 2015)
GSH	Freezing	Frozen	2 mM	Increases farrowing rates and the number of total of born-alive piglets	(Estrada *et al.*, 2014)
GSH	Freezing	Frozen	2 mM	Decreases DNA fragmentation	(Yeste *et al.*, 2013)
GSH	Freezing	Frozen	2-5 mM	GSH 2 mM improves cryotolerance of semen but poor freezability ejaculates need a higher concentration (5mM)	(Yeste et al., 2014)
GSH	Freezing	Frozen	1 mM and 5 mM	Improvement in sperm motility, viability and non-capacitated viable spermatozoa	(Gadea *et al.*, 2005)
GSH	Thawing	Frozen	5 mM	Increases sperm fertilizing ability	(Gadea *et al.*, 2004)
GSH		Liquid	5 mM	Improves sperm viability and acrosome integrity	(Funahashi and Sano, 2005)
L-Cysteine		Liquid	5 mM	Improves sperm viability, acrosome integrity and IVF outcome	(Funahashi and Sano, 2005)
L-Cysteine	Freezing	Frozen	5 or 10 mmol L^{-1}	Increases progressive motility, viability and acrosome integrity of thawed semen	(Kaeoket *et al.*, 2010)

Antioxidant	Point of antioxidant addition	Type of semen storage	Antioxidant concentration	Principal effects of antioxidant	Reference
L-Cysteine	Freezing	Frozen	5 mM L^{-1}	Improves post-thawed semen progressive motility and acrosome integrity	(Chanapiwat *et al.*, 2009)
Lutein	Freezing	Frozen	10 µM (Lutein)	Lutein and Trolox + AA improve resilience to osmotic stress and motility and decreases DNA fragmentation	(Varo-Ghiuru *et al.*, 2015)
Trolox + AA			400+200 µM (Trolox +AA)		
Melatonin		Liquid (3-12h)	100 nM	Increases sperm motility, viability, survival rates, membrane integrity, in vitro embryo development and decreases LPO	(Jang *et al.*, 2010)
Melatonin		Liquid (17°C for 7 days)	1 µM	Increases the proportion of live sperm with an intact acrosome, but is not able to improve the spermatic quality	(Martín-Hidalgo *et al.*, 2011)
***Rhodiola sacra* aqueous extract (RSAE)**	Freezing and thawing	Frozen	4-8 mg/L	Improves sperm motility and GSH and MAD concentrations	(Zhao *et al.*, 2009)
Rosemary	Freezing	Frozen	2.5-5-10 g/100mL	Improves the post-thaw epididymal boar spermatozoa motility and prevents peroxidation	(Malo *et al.*, 2011)
Rosmarinic acid	Freezing	Frozen	105 µM	Improves motility, plasma and acrosomal membrane integrity, penetration rate; descreases DNA oxidation rate	(Luño *et al.*, 2014)
SOD	Freezing	Frozen	150-300 UI/mL	Increases post-thaw viable sperm	(Roca *et al.*, 2005)
Trolox (Vitamin E analogue)	Freezing	Frozen	200 µM	Improves post-thawing sperm motility and mitochondrial activity	(Peña *et al.*, 2003)
α-tocopherol (Vitamin E)	Freezing	Frozen	200 mg/mL	Protects spermatozoa against lipid peroxidation	(Breininger *et al.*, 2005)
α-tocopherol (Vitamin E)	Freezing	Frozen	200 mg/mL	Enhances sperm motility; reduces capacitation-like changes and tyrosine phosphorylation	(Satorre *et al.*, 2007)
α-tocopherol (Vitamin E)	Freezing	Frozen	200 µM	Reduces lipid peroxidation and the expression of apoptosis genes	(Jeong *et al.*, 2009)
α-tocopherol (Vitamin E)	Freezing and thawing	Frozen	1 mM	Protects against DNA fragmentation	(Casey *et al.*, 2011)
β-mercaptoethanol	Thawing	Frozen	25 or 50 µM	Inhibits sperm capacitation and spontaneous acrosome reaction; litter size after AI tends to be higher	(Yamaguchi and Funahashi, 2012)

Green tea polyphenols (GT-PFs) and Epigallocatechin-3-gallate (EGCG)

Tea, derived from *Camellia sinensis* leafs, is one of the most common beverage in the world. From ancient times, green tea has been known in the traditional Chinese medicine for its healthy proprieties, higher than those of black tea ones (Chacko *et al.*, 2010). These beneficial effects are mainly attributed to a higher polyphenols (PFs) content than black tea (see *Table 2*) (Khan and Mukhtar, 2007; Chacko *et al.*, 2010).

Table 2 *- Principal flavonoid components of green and black tea beverage in comparison (% wt/wt solids) (Balentine et al., 1997, modified).*

Flavonoid (Polyphenols)	Green tea	Black tea
Catechins	30-42	3-10
Theaflavins	/	2-6
Simple polyphenols	2	3
Flavonols	2	1
Other polyphenols	6	23
Theanine	3	3
Aminoacids	3	3
Peptides/protein	6	6
Organic acids	2	2
Sugars	7	7
Other carbohydrates	4	4
Caffeine	3-6	3-6
Potassium	5	5
Other minerals/ash	5-8	5-8

PFs are natural low weight molecules produced in high concentration when green plants undergo to physical/chemical/biochemical stress in order to defence themselves (Afzal *et al.*, 2015). Some studies reported that polyphenols are effective scavengers of reactive oxygen species (ROS) and can indirectly act on transcription factors and enzyme activities as antioxidants (Higdon and Frei, 2003).

The most important PFs in green tea leafs are flavonoids (2-phenylbenzopyran) where flavanols and flavonols are the main representative classes (30% of the entire fresh leaf dry weight) (Balentine *et al.*, 1997). Many biological proprieties of green tea are probably due to polyphenolic flavanols named catechins (Afzal *et al.*, 2015). They can be found also in black tea but, during its manufacturing, catechins undergo a high reduction (Balentine *et al.*, 1997); as reported in *Table 2,* the concentration of catechins amount to 30-42% vs 3-10% (wt/wt) in green and black tea, respectively.

Catechins are molecules composed by a 2-phenylchromane skeleton substituted in many terminal positions by hydroxyl groups (–OH) (Khan and Mukhtar, 2007).

Four are the major catechins (flavan-3-ols) found in green tea: (-)-epigallocatechin gallate (EGCG), (-) epigallocatechin (EGC), (-)-epicatechin gallate (ECG) and (-)-epicatechin (EC) (Khan and Mukhtar, 2007) (*see Figure 4*).

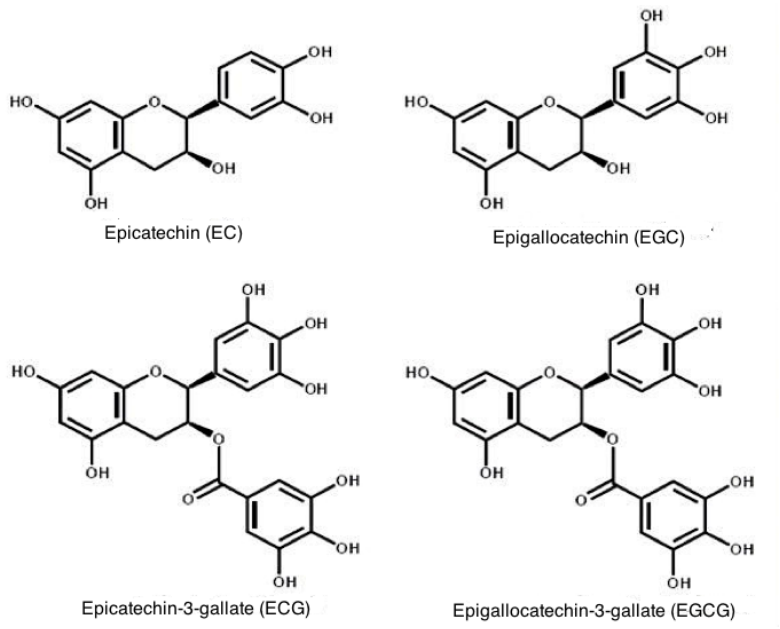
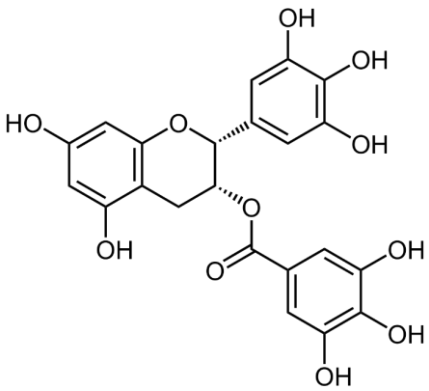


Figure 4 *- Major tea catechins (Gullo and Carlomagno, 2010, modified).*

Among catechins, only Epigallocatechin-3-gallate (EGCG), that represents 50-80% of all catechins in green tea (Khan and Mukhtar, 2007), is of particular interest in medical chemistry for its proprieties. The name "gallate" in EGCG is due to the fact that it is a catechin with the –OH group in 3' position esterified with gallic acid, while the term "epi" indicates that it is a levorotatory compound (Khan and Mukhtar, 2007; Legeay *et al.*, 2015) (*see Figure 5*).



Epigallocatechin-3-3gallate (EGCG)

Figure 5 *- Molecular structure of Epigallocatechin-3-gallate (Legeay et al., 2015, modified).*

Thanks to the large number of –OH groups in molecular structure (that characterizes all catechins) and gallate in C-3 position, EGCG is the best free radical scavenger of all catechins because (1) its capability for giving electrons, conferring high reactivity to quench free radicals and (2) its ability to chelate metal ions by dihydroxy or trihydroxyle structure, preventing free radicals generation (Khan and Mukhtar, 2007; Legeay *et al.*, 2015). For these two reasons, EGCG is considered as one of the most active molecules known for antioxidant properties (Higdon and Frei, 2003; Afzal *et al.*, 2015; Legeay *et al.*, 2015), higher than those of other molecules with good antioxidant activity as vitamins C or vitamin E (Zhao *et al.*, 1989; Kaedei *et al*., 2012).

Oxidative damage is known to be implicated in the pathogenesis of a great number of chronic diseases, as cardiovascular diseases, cancers and neurodegenerative syndromes among which oxidative stress plays a key role in its development and progression (Higdon and Frei, 2003) and where antioxidant molecules, like catechins (and polyphenols in general), could help to fight these illnesses.

In human, EGCG is a molecule well studied, especially in the last few decades, and now it is suitable for many purposes. For example, literature reported that EGCG has anti-inflammatory (Tipoe *et al.*, 2007), cardioprotective (Afzal *et al.*, 2015), hypoglycemic, hypercholesteremic (Yousaf *et al.*, 2014), anticancer anti-angiogenetic and neuroprotective effects (Rahmani *et al.*, 2015; Xicota *et al.*, 2015). EGCG antioxidant effects are also studied in gametes, which are more exposed to oxidative stress under laboratory condition than in vivo.

An optimal semen preservation is very important to improve both IVF and AI. EGCG could act positively on porcine species: if frozen-thawed boar spermatozoa are co-incubated in a IVF medium supplemented with EGCG, sperm motility and oocytes penetration rate are both improved, although the effects vary between individual males (Kaedei *et al.*, 2012). Moreover, EGCG seems to exert an inhibition on caspase activation (decreasing dead cells number) protecting sorted spermatozoa from the detrimental effects of storage (Vallorani *et al.*, 2010).

It is important to keep in mind that EGCG can also works as a pro-oxidant molecule in certain condition or in certain concentration, leading to auto-oxidation (as Resveratrol*, see chapter dedicated*). It can produce hydrogen peroxide (Furukawa *et al.*, 2003) that could damage DNA and sperm plasma membrane via lipid peroxidation (Chen *et al.*, 1997; Kemal Duru *et al.*, 2000; Kusakabe and Kamiguchi, 2004).

Literature also reported that in dog, ram, rat and rooster the extender supplementation during semen storage with green tea polyphenols extracts (GT-PFs extracts), where EGCG is one of the constituents and the major representative catechin, lead to an improve in semen quality in terms of viability and/or motility (Al–Daraji, 2011; Wittayarat *et al.*, 2012, 2013; Dias *et al.*, 2014; Husam J. H. Banana, 2015).

Tables 3-4 summarize recent studies on the effects of Epigallocatechin-3-gallate (EGCG) or Green tea polyphenols extracts (GT-PFEs) or Green/White Tea extracts (GTEs/WTEs) on spermatozoa of different species.

Table 3 *- Recent studies on the effects of Epigallocatechin-3-gallate (EGCG) or Green tea polyphenols extracts (GT-PFEs) or Green/White Tea extracts (GTEs/WTEs) on liquid storage spermatozoa of different species.*

Male	Effect of EGCG or GT-PFs extracts on liquid storage spermatozoa	EGCG/GT-PFs/GTEs/WTEs concentration	Reference
Boar	↑ viability	40 µg/mL EGCG	(Vallorani *et al.*, 2010)
Dog	↑ viability	0.75 mg/mL GT-PFs	(Wittayarat *et al.*, 2012)
	↑ motility		
Dog	↑ viability	0.5-0.75-1 mg/mL GT-PFs	(Wittayarat *et al.*, 2013)
	↑ motility		
Man	↓ HIV transmission rate by ejaculation	0.4 µM EGCG	(Hartjen *et al.*, 2012)
Ram	↑ viability	2-4-6 mL/100mL GTEs	(Husam J. H. Banana, 2015)
	↑ motility		
	↓ abnormalities		
Rooster	↑ viability	6-8-10-12 mL/100mL GTEs	(Al–Daraji, 2011)
	↑ motility		
	↓ abnormal spermatozoa		
	↑↑ viability and motility	10 ml/100 mL GTEs	
	↓↓ abnormalities		
Mouse	↑ sperm chromatin damage	10 µM EGCG	(Kusakabe and Kamiguchi, 2004)
Rat	↑ viability	0.5-1 mg/mL GTEs 0.5-1 mg/mL WTEs	(Dias *et al.*, 2014)
	↑ spermatozoa antioxidant potencial		
	↓ lipid peroxidation		
Stallion	↑ zona pellucida binding	10-20 µM EGCG	(Plaza Dávila *et al.*, 2015)

Table 4 *- Recent studies on the effects of Epigallocatechin-3-gallate (EGCG) or Green tea polyphenols extracts (GT-PFEs) or Green/White Tea extracts (GTEs/WTEs) on frozen storage spermatozoa of different species.*

Male	Effect of EGCG on frozen spermatozoa	EGCG/GT-PFs concentration	Reference
Boar	↓ lipid peroxidation	5% GTEs	(Gale *et al.*, 2015)
Boar	↑ post-thaw viability	0.01 % w/v PFs	(Kitaji *et al.*, 2015)
	↑ monospermic oocytes		
	↑ blastocystic formation		
Boar	↑ motility ↑ penetration rate into oocytes	50 µM EGCG	(Kaedei *et al.*, 2012)

Resveratrol (RESV)

Resveratrol (3,4',5-trihydroxystilbene; RESV) is a phytoalexin with a stilbene structure isolated from the roots of white hellebore in 1940; later it was identified as an active ingredient from *Polygonum cuspidatum* (Gescher, 2008). This plant is used in oriental medicine in form of dried and powdered roots for many illness treatments (dermatitis, arteriosclerosis, inflammatory disease) (Arichi *et al.*, 1982; Siemann and Creasy, 1992).

RESV is synthetized by leaf tissues and in the last years it has been identified in a lot of plant species; it protects them from parasitic attacks (in particular from fungal infections) or environmental stress (like ultraviolet light exposure). Among all the plants, grapes have the major content of RESV; also, wine is rich in that this molecule is specifically found in grape seed and skin (Siemann and Creasy, 1992; Fernández-Mar *et al.*, 2012). However, RESV is widely consumed in the Mediterranean diet in form of plums and peanuts too (Fernández-Mar *et al.*, 2012).

The interest in RESV molecule began in 1992 when it was associated to the red wine cardio-protective effect (Siemann and Creasy, 1992; Gescher, 2008).

RESV is composed by two aromatic rings connected by a styrene double bond (*Figure 6*).

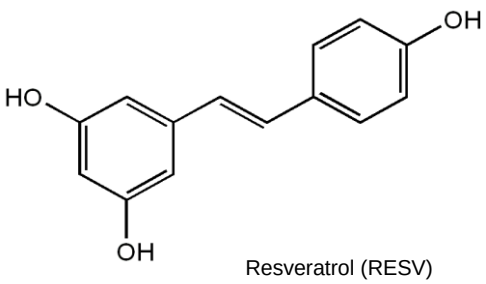


Figure 6 *- Chemical structure of 3,4',5-trihydroxystilbene (RESV) (Pirola and Fröjdö, 2008, modified).*

The interest for RESV in medicine researches depends on its particular structure: the number and position of its hydroxyl groups (OH), the intramolecular hydrogen bonds, the stereo-isometry and the presence of double bonds give to the molecule many biological properties (Ovesná and Horváthová-Kozics, 2005). RESV is reported to have anti-inflammatory, immunomodulation, cardio-protective and anti-apoptotic effects as well as anticancer activity (Gusman *et al.*, 2001; Pervaiz and

Holme, 2009) because it can interact with a great number of enzymes (kinases, lipo and cyclooxygenases, sirtuins and transcriptor factors) involved in important intracellular signalling pathways (Pirola and Fröjdö, 2008); in particular, sirtuin 1 (SIRT1), plays a role in cellular energy homeostasis and mitochondrial biogenesis (Itami *et al.*, 2015).

Another very important structure in RESV molecule is the 4-hydroxystilbene element, which is the responsible for the antioxidant properties (Pervaiz and Holme, 2009; Queiroz *et al.*, 2009). In this way RESV can act as antioxidant thanks to its ability to reduce mitochondrial ROS production, to inhibit lipids peroxidation, to scavenge superoxide radicals and to regulate antioxidant enzymes and cofactors expression (Pervaiz and Holme, 2009).

In recent years, many studies using RESV as antioxidant in order to protect spermatozoa from detrimental effects of oxidative stress during semen storage have been performed (as discussed in previous chapters). In mouse (Mojica-Villegas *et al.*, 2014), bovine (Tvrdá *et al.*, 2015a) and human (Collodel *et al.*, 2011) RESV could protect spermatozoa from experimentally induced oxidative stress and it is effective in ROS reduction, decreasing mitochondrial membrane potential and capacitation-like changes in ram and buffalo sperm (Silva *et al.*, 2012; Longobardi *et al.*, 2017). RESV can also prevent lipid peroxidation in human and boar spermatozoa (Garcez *et al.*, 2010; Federico *et al.*, 2012; Rigo *et al.*, 2017) and DNA damages caused by cryopreservation in human and bull sperm (Branco *et al.*, 2010; Bucak *et al.*, 2015).

As you can see in the table below, RESV does not always have beneficial effects on spermatozoa, which on the contrary depend on animal species, type of semen storage and antioxidant concentration used.

The results of recent studies on the effects of RESV recorded on spermatozoa of different species are summarized in the *Table 5-6*.

Table 5 *- Recent studies on the effects of Resveratrol (RESV) on liquid storage spermatozoa of different species.*

Male	Effect of RESV on liquid storage spermatozoa	RESV concentration	Reference
Boar	↓ ATP content	33 and 100 µM	(Martín-Hidalgo *et al.*, 2013)
	↓ mmp (mitochondrial membrane potential)	10-33-66-100 µM	
	↓ motility	100 µM	
	↓ response to capacitating stimuli	33 and 100 µM	
Boar	↓ lipid peroxidation	1 mM	(Rigo *et al.*, 2017)
	↑ capacitation-like changes (↑ membrane fluidity)		
Bull	Preservation of viability	10-25-50 µM/L (-1)	(Tvrdá et al., 2015)
Bull	↑ acrosome reaction	7.5-15-30 µg/mL	(Assunção *et al.*, 2015)
Bull	Preservation viability ↓ ROS	10-50 µM/L	(Tvrdá *et al.*, 2015b)
	↓ viability ↓ motility	100-200 µM/L	
Man	↑ motility	6-15-30 µM	(Collodel *et al.*, 2011)
	preserves sperm chromatin	15 µM	
	↓ lipid peroxidation		
Man	↓ oxidative stress damages (lipid peroxidation)	15 µM	(Federico *et al.*, 2012)
Mouse	Maintain fertility	15 µg/mL	(Mojica-Villegas *et al.*, 2014)
	Protection from lipid peroxidation		
Stallion	↓ viable sperm with high mmp	40 and 80 mM	(Giaretta *et al.*, 2014)
	↓ total and progressive motility	80 mM	

Table 6 *- Recent studies on the effects of Resveratrol (RESV) on cryo-preserved spermatozoa of different species.*

Male	Effect of RESV on cryopreserved spermatozoa	RESV concentration	Reference
Buffalo	↓ capacitation-like changes	50 µM	(Longobardi *et al.*, 2017)
	↑ IVFertilizing ability		
Buffalo	↓ low capacitation level	50 µM	(Longobardi *et al.*, 2015)
Bull	↑ mitochondrial activity	1 mM	(Bucak *et al.*, 2015)
	↑ motility		
	↓ DNA damage		
Man	↑ AMP-activated protein kinase phosphorylation	15 µM	(Shabani Nashtaei *et al.*, 2017)
	↑ mmp		
	↓ ROS		
	↓ apoptosis-like changes		
Man	↓ DNA damages	10 mM	(Branco *et al.*, 2010)
Man	Prevent post-thawing lipo-peroxidation	0.1, 1.0 and 10 mM	(Garcez *et al.*, 2010)
Ram	↓ mmp	20 µg/mL	(Silva *et al.*, 2012)
Stallion	↓ viability	1 and 10 mM	(Matheny *et al.*, 2015)
	↓ motility		
	↓ ROS		
	↑ DNA fragmentation	1 mM	

SEMEN PHOTO-STIMULATION

Photo-stimulation consists in irradiating cells with light in order to modify their metabolism. Since 1980s, the biological effects of photo-stimulation were studied in many tissues and also in gametes (Abdel-Salam and Harith, 2015). Laser irradiation can increase the production of adenosine triphosphate (ATP) thus consequently increasing the energy provided to the cell. Since sperm motility depends on energy consumption, an increase in the energy supply to the cells is of great importance.

The first work on spermatozoa irradiation was performed in 1969 when Goldstein et al. light-stimulated starfish and sea-urchin spermatozoa using pulsed ruby laser micro-beam (Goldstein, 1969). Further studies focused on the light-effects on mammalian sperm cells using different types of low-power lasers. In summary, photo-stimulation has been found to: (1) increase cell energy supply via ATP; (2) improve motility of fresh semen; and (3) control the level of bacterial contamination in semen doses (reviewed by Abdel-Salam and Harith, 2015)

With regard to frozen-thawed sperm, studies conducted in different species have provided interesting results. For example, Helium-Neon (He-Ne) laser irradiation on cryopreserved avian sperm restored better their motility at post-thaw (Iaffaldano *et al.*, 2013). In rams, photo-stimulation of frozen-thawed sperm maintained better sperm motility, viability and mitochondrial function (Nicolae *et al.*, 2015); in addition, a clear interaction between mitochondria and laser-light was observed (Iaffaldano *et al.*, 2016). In bulls, low-laser irradiation of frozen-thawed sperm also preserved better their viability and acrosome integrity, even when incubated under capacitating conditions (Ocaña-Quero *et al.*, 1997; Fernandes *et al.*, 2015). Iaffaldano *et al.*, 2010 showed that He-Ne laser irradiation improved rabbit sperm preservation during liquid storage by modulating sperm qualitative functions. This effect may be related to the evidence of energetic bio-stimulation of rabbit spermatic cells and to an improved cytochrome c oxidize activity. Moreover, Corral-Baqués *et al.*, 2005 found that irradiating dog sperm with a 655nm diode laser light at 4.00, 6.00, and 10.00 J/cm^2 improves its motility parameters and seems to maintain its functional characteristics up to 45 min after irradiation. In 2008 the same research group (Corral-Baqués *et al.*, 2008) extended its investigations on the effects of low-level laser irradiation on dog spermatozoa and its dependence on the laser output power. The results showed that irradiation with different output powers had different effects on semen parameters including motility, average velocity, linear coefficient and beat cross frequency. Yazdi *et al.*, 2014 reported the effects of 830 nm diode laser irradiation on

human sperm motility. Significant increases in the irradiated sperm motility were observed after exposure to 4.00 and 6.00 J/cm^2 for 60 and 45 min, respectively.

The effects of photo-stimulation have been studied also in pig liquid-stored sperm. The exposure of liquid-stored boar semen to light emission diode (LED)-based red light (wavelength range: 620nm-630nm) improves, perhaps via a mechanism related to mitochondrial function, sperm motility and mitochondrial membrane potential, and increases the fertilizing ability of boar seminal doses (Yeste *et al.*, 2016). In this context, it is worth noting that the aforementioned study did not use a laser as light-source but rather LED, as this system is cheaper and easier to use than conventional lasers. In this way, the exposure of semen to light could be performed not only in laboratory conditions but also in the daily routine of commercial AI centers.

OBJECTIVES

Handling of semen is very important for a successful artificial insemination in both pig and horse farming. Liquid storage and cryopreservation are the techniques routinely utilized for male gamete storage. One of the main problems during liquid and frozen semen storage is the loss of fertilizing potential due to “cold-shock”, a variety of harmful cellular alterations mainly related to an excessive production of reactive oxygen species (ROS) and alterations in antioxidant defense systems. This phenomenon is called oxidative stress. It is known that ROS play an important role in the process of sperm maturation and appropriate levels are essential for sperm hyperactivation and capacitation. However, ROS over-production could be detrimental for sperm cells due to the presence of high proportions of unsaturated fatty acids in their cell membrane and scarce cytoplasm, which consequently results in an inadequate amount of antioxidant enzymes and low protection against ROS. So far, many studies have been performed to improve the efficiency of sperm liquid and cryopreservation techniques, in order to increase the percentage of viable cells after storage and to enhance their quality and fertilizing ability. Supplementation of sperm preparation with different antioxidants gave interesting and promising results and, during recent years, use of plant antioxidants has been gaining attention of several research groups.

Moreover, an increase in the fertilising ability of boar sperm has been demonstrated after photo-stimulation.

On these bases, the objective of the present thesis was to assess whether:

- Resveratrol (RESV) or Epigallocatechin-3-gallate (EGCG) supplementation of thawing boar semen extender is effective in influencing sperm quality parameters and in vitro fertilization ability (IVF) (***Papers 1-2***);
- EGCG and green tea extract polyphenols (GT-PFs) improve stallion semen parameters during cooling at 4°C (***Paper 3***);
- Photo-stimulation improves frozen-thawed boar semen quality (***Paper 4***).

RESULTS

The results are briefly reported below, while the whole papers are in the following chapter (*"Papers Compendium"*):

- **PAPER 1:**

  "Resveratrol and Epigallocatechin-3-gallate addition to thawed boar sperm improves in vitro fertilization."

  The objective of this first paper was to assess the effect of different concentrations of RESV (0.5, 1, and 2 mM) or EGCG (25, 50 and 100 µM) added for 1 h at 37°C to thawing boar semen extender. Both antioxidants, even if not able to exert any effect on sperm quality parameters (viability and acrosome integrity), efficiently improved in vitro penetration rate at all the concentrations tested. Moreover, the highest concentration of RESV (2mM) and the first two low doses of EGCG (25 and 50 µM) significantly increased the total efficiency of IVF.

- **PAPER 2:**

  "Combined effects of Resveratrol and Epigallocatechin-3-gallate on post thaw boar sperm and IVF parameters."

  The aim of the second paper was to study the effect of supplementing boar sperm thawing medium with most powerful concentrations of EGCG (50 µM) and RESV (2 mM) tested in the first paper, alone or in combination, on in vitro fertilization (IVF) and to study the effect on boar sperm motility (assessed by CASA), viability, acrosome integrity, mitochondrial function, lipid peroxidation and DNA integrity (assessed by flow cytometry) and protein tyrosine phosphorylation (assessed by immunofluorescence).

  Our results demonstrate that viability, acrosome integrity, mitochondrial functionality and lipid peroxidation are not influenced by the addition of the antioxidants. DNA integrity is negatively influenced by RESV (both alone or associated with EGCG) both at 1 and 4 h incubation. Sperm motility is negatively affected by RESV (alone or associated with EGCG) in comparison to control and EGCG groups both at 1 and 4 h; this effect is evident both in average motility parameters and in single cells kinematics (studied by cluster analysis). Tyrosine phosphorylated protein immunolocalization, used as capacitation parameter, is not affected by the different treatments. Finally, penetration rate is strongly enhanced by RESV, both alone or associated with EGCG; EGCG increases penetration rate as well but to a lower extent.

- **PAPER 3:**

  "Epigallocatechin-3-gallate (EGCG) and green tea polyphenols do not improve stallion semen parameters during cooling at 4°C."

  The aim of the third work was to evaluate the effect of two natural antioxidants (EGCG and green tea extract polyphenols) on some sperm parameters (sperm motility, viability/acrosome integrity and DNA quality) in extended semen immediately after its collection (T0) and after 2, 6, 24 and 48 h of cool storage. No beneficial effect on the analyzed parameters was observed as the two antioxidants were not able to improve sperm quality during 48 h of storage.

- **PAPER 4**:

  "Photo-stimulation through LED prior to cryopreservation improves the cryotolerance of poor freezability boar ejaculates."

  This last study sought to address whether photo-stimulation of boar sperm before cryopreservation could increase their cryotolerance and, thus, positively affect their function and survival. LED photo-stimulation procedures increased the resilience of poor freezability ejaculates to withstand cryopreservation, especially when viability, acrosome integrity and mitochondrial activity were evaluated at 180 minutes post-thaw. On the contrary, photo-stimulation had no effect on good freezability ejaculates and a detrimental effect on total sperm motility was observed in both types of ejaculates.

PAPERS COMPENDIUM

PAPER 1

"Resveratrol and Epigallocatechin-3-gallate addition to thawed boar sperm improves in vitro fertilization"

NOTICE:

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Revised

Resveratrol and Epigallocatechin-3-gallate addition to thawed boar sperm improves in vitro fertilization

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Abstract

Thawing is one of the most delicate process after semen cryopreservation as spermatozoa pass from a dormant metabolic stage to a sudden awakening in cellular metabolism. The rapid oxygen utilization leads to an overproduction of reactive oxygen species that can damage sperm cells, thus causing a significant decrease of fertilizing potential of frozen-thawed spermatozoa. Resveratrol (Res) is a natural grape-derived phytoalexin and Epigallocatechin-3-gallate (EGCG) is the major polyphenol in green tea (*Camellia sinensis*); both molecules are known to possess high levels of antioxidant activity. The objective of the present study was to assess the effect of different concentrations of Res (0.5, 1 or 2 mM; Experiment 1) or EGCG (25, 50 or 100 μM; Experiment 2) supplementation to thawing boar semen extender on sperm quality parameters (viability and acrosome integrity) and in vitro fertilization (IVF).

Semen after thawing and dilution with three volumes of Beltsville Thawing Solution (BTS), was immediately divided in control group without antioxidants addition (CTR) and either Res or EGCG groups. Sperm viability and acrosome integrity were evaluated in CTR, Res or EGCG groups after 1 h of incubation at 37°C.

The addition of different doses of Res or EGCG to thawing extender for 1 h did not induce any effect on boar sperm viability and acrosome integrity. However, both Res and EGCG treated samples exhibited a significantly higher penetration rate compared with CTR when used for IVF. In particular the treatment with all the EGCG concentrations increased the penetration rate ($P< 0.01$) while only Res 2 mM induced a significant increase of this parameter ($P< 0.01$). In addition, EGCG 25 and 50 μM supplementation significantly increased total fertilization efficiency as compared to control (EGCG 25 μM: 40.3 ± 8.2 vs 26.8 ± 9.5, $P< 0.05$; EGCG 50 μM: 40.4 ± 7.8 vs 26.8 ± 9.5, $P < 0.01$). The same effect was observed with Res 2 mM (51.0 ± 7.6 vs 29.6 ± 11.3, $P<0.01$).

In conclusion, our results indicate that the addition of different doses of the two antioxidants to thawed spermatozoa for one hour, even if does not exert any effect on sperm viability and acrosome integrity, efficiently improves in vitro penetration rate. Moreover, both molecules (EGCG 25 and 50 μM and Res 2 mM) significantly increases the total efficiency of fertilization.

Key words: boar semen, cryopreservation, antioxidants, fertilization, Resveratrol, Epigallocatechin-3-gallate

1. Introduction

Sperm cryopreservation is the most efficient method for long term sperm storage (reviewed in [1]). However, frozen-thawed boar semen is not routinely used because of the high performance of long-term extenders for liquid storage and the non optimal quality of thawed boar spermatozoa. Anyhow it is important to create an efficient cryopreserved semen gene bank, planning insemination at artificial insemination centers, maintaining genetic diversity and promoting the rapid growth of swine models [2,3].

During the cryopreservation process, spermatozoa undergo a variety of harmful cellular alterations called “cold shock”, mainly induced by the increase of reactive oxygen species (ROS) levels [4,5]. ROS, such as hydrogen peroxide (H_2O_2), superoxide anions (O_2^-), hydroxyl radicals (OH^-), generated during intermediate steps of oxygen reduction, are known for their ability to damage cellular proteins, DNA and plasma membrane lipids, due to their free radical nature [6]. Even if very low and controlled concentrations of ROS are required for sperm hyperactivation, capacitation, acrosome reaction and zona binding events [7–9], when ROS are overproduced, spermatozoa cannot easily adapt to this condition and oxidative stress occurs leading to cell damage [6].

On these bases, frozen-thawed boar spermatozoa may present nucleoprotein-DNA structural alterations [8,10–12] and capacitation-like changes that could lead to an important reduction in fertilizing potential of frozen-thawed sperm [13]. In order to reduce oxidative damage, one approach is to supplement semen extender with enzymatic and non-enzymatic antioxidant compounds during freeze-thawing.

Resveratrol (3,4’,5-trihydroxy-trans-stilbene) (Res) is a polyphenolic natural product with a stilbene structure isolated at first from the roots of white hellebore in 1940 [14] and later from Polygonum cupsidatum, a medicinal plant. Today it is widely consumed in the Mediterranean diet in the form of peanuts, grapes and wine. Res shows many biological activities such as anti-inflammatory, cardioprotective, chemopreventive and antiapoptotic [15,16]. Moreover, Res has been reported to possibly act as antioxidant thanks to its ability to reduce mitochondria ROS production, scavenge superoxide radicals, inhibit lipid peroxidation, and regulate the expression of antioxidant cofactors and enzymes [16]. It has been reported that mouse [17], bovine [18] and human spermatozoa [19] can be protected by Res from experimentally induced oxidative stress. A protective effect of Res against membrane oxidative damage but not against the loss of motility induced by the cryopreservation of human semen, has been observed [20]. Furthermore, Res is effective in minimizing post-thawing DNA damage in human spermatozoa [21] and in improving post-thaw bull sperm quality in terms of sperm motility, high mitochondrial activity and DNA integrity [22]. In

frozen-thawed ram sperm the addition of Res to the Tris-egg yolk-glycerol extender has been shown to reduce sperm mitochondrial membrane potential [23].

Epigallocatechin-3-gallate (EGCG) is the major polyphenol in green tea (*Camellia sinensis*) and is reported to possess a high level of antioxidant activity [24,25]. The supplementation of canine sperm with green tea polyphenol extracts (PFs) as been demonstrated to improve motility and viability of spermatozoa during long-term liquid storage [26,27]. Moreover, pre-treatment of boar spermatozoa with PFs prior to freezing exhibited significantly higher degrees of post-thaw sperm viability and acrosomal integrity [28]. The beneficial effect of EGCG has been observed during liquid storage at 15°C of sorted boar semen: it increased the percentage of viable spermatozoa and inhibited caspase activation [29].

On these bases, the objective of the present study was to assess whether Res or EGCG supplementation of thawing boar semen extender is effective in influencing sperm quality parameters (viability and acrosome integrity) and in vitro fertilization (IVF).

2. Materials and Methods

Unless otherwise specified, all the reagents were purchased from Sigma–Aldrich (Milan, Italy).

2.1 Sperm thawing

The study was performed using commercial frozen semen from 3 Large White boars purchased by Suiseme Srl (Saliceta San Giuliano, Modena, Italy). Straws (0.5 mL/straw) were thawed for 30 sec in water bath at 37ºC and immediately diluted, at the same temperature, with three volumes of Beltsville Thawing Solution (BTS).

Semen was immediately divided in the following experimental groups: CTR (control: without antioxidant addition), and either Res (addition of 0.5, 1, 2 mM Res to BTS thawing extender; Experiment 1) or EGCG (addition of 25, 50, 100 µM EGCG to BTS thawing extender; Experiment 2). Only sperm samples with viability > 40% as assessed immediately after thawing were used for the experiments.

Sperm viability and acrosome integrity were evaluated 1 h after thawing in CTR and Res or EGCG groups. After 1 h of incubation at 37°C in either absence or presence of different doses of Res or EGCG, semen samples were washed and used for in vitro fertilization (IVF).

98 *2.2 Post-thaw spermatozoa evaluation*

99 *2.2.1 Sperm viability assessment*

100 Sperm viability was evaluated by incubating 25 µL of semen with 2 µL of a 300 µM Propidium Iodide
101 (PI) stock solution and 2 µL of a 10 µM SYBR-14 stock solution (LIVE/DEAD®Sperm Viability kit,
102 Molecular Probes, Invitrogen), for 5 min at 37°C in the dark. After incubation, 10 µL of sperm
103 suspensions were analyzed with a Nikon Eclipse epifluorescence microscope using a double-band-
104 pass filter for green and red fluorescence. The spermatozoa with green or red fluorescence on the
105 head were considered live or dead, respectively (see supplementary file, panel A). At least 200 cells
106 were counted in each analysis.

107 *2.2.2 Acrosome integrity assessment*

108 Acrosome integrity was measured with a FITC conjugated lectin from Pisum Sativum (FITC-PSA)
109 which labels acrosomal matrix glycoproteins. Spermatozoa were washed twice in PBS, resuspended
110 with ethanol 95% and fixed at 4°C for 30 min. Samples were dried in heated slides and incubated
111 with FITC-PSA solution (5.0 µg PSA-FITC/1 mL H2O) for 20 min in darkness. After staining
112 samples were washed in PBS and mounted with Vectashield mounting medium with PI (Vector
113 Laboratories, Burlingame, CA, USA). The slides were then observed with the above described
114 fluorescence microscope. The presence of a green acrosomal fluorescence was considered indicative
115 of an intact acrosome, while a partial or total absence of fluorescence was considered to indicate
116 acrosome disruption or acrosome reaction (see supplementary file, panel B).

117 *2.3 In vitro maturation (IVM) of cumulus-oocyte-complexes*

118 Ovaries were collected at a local abattoir and transported to the lab within 2 h in a thermos filled with
119 physiological saline at 30-35°C. Cumulus oocyte complexes (COCs) from follicles 3-6 mm in
120 diameter were aspirated using 18 gauge needle attached to a 10 mL disposable syringe. Under a
121 stereomicroscope, intact COCs were selected and transferred into a petri dish (35 mm, Nunclon,
122 Roskilde, Denmark) prefilled with 2 mL of modified PBS supplemented with 0.4% BSA. Only COCs
123 with complete and dense cumulus oophorus were used. After three washes in NCSU 37 [30]
124 supplemented with 5.0 mg/mL insulin, 0.57 mM cysteine, 10 ng/mL epidermal growth factor, 50 µM
125 ß-mercaptoethanol and 10% PCV2-PCR-negative porcine follicular fluid (IVM medium), groups of
126 50 COCs were transferred to a Nunc 4-well multidish containing 500 µl of the same medium per well
127 and cultured at 39°C in humidified atmosphere of 5% CO_2/7% O_2 in air. For the first 22 h of *in vitro*

maturation, the IVM medium was supplemented with 1.0 mM dibutyryl cyclic adenosine monophosphate (db-cAMP), 10 IU/mL eCG (Folligon, Intervet, Boxmeer, The Netherlands) and 10 IU/mL hCG (Corulon, Intervet). For the last 22-24 h COCs were transferred to fresh maturation medium [31].

2.4 In vitro fertilization (IVF)

For in vitro fertilization, thawed semen after 1 h of incubation at 37°C with or without antioxidants, was washed twice with BTS and finally resuspended with Brackett & Oliphant's [32] medium supplemented with 12% foetal calf serum (Gibco, Invitrogen, Italy) and 0.7 mg/mL caffeine (IVF medium). Sperm concentrations were evaluated and 45–50 matured oocytes, freed from cumulus cells by gentle repeated pipetting, were transferred to 500 mL IVF medium containing 1 x 10^6 sperm/mL. After 1 h of co-culture, oocytes were transferred to fresh IVF medium and cultured for 19 h until fixation in acetic acid/ethanol (1 : 3) for 24 h and stained with Lacmoid.
The oocytes were observed under a phase-contrast microscope and the following parameters were assessed:

(1) penetration rate (number of fertilized oocytes/ number of inseminated oocytes);

(2) monospermy rate (number of oocytes containing only one sperm head–male pronucleus/ number of penetrated oocytes);

(3) total efficiency of fertilization (number of monospermic oocytes/number of inseminated oocytes).

Degenerated and immature oocytes were not counted.

2.5 Statistical analysis

Data were analyzed using R version 3.0.3 [33]. Significance was set at $p<0.05$ unless otherwise specified. Results are expressed as mean ± standard deviation. One-way ANOVA and Tukey post hoc test were performed to assess the difference in sperm viability and acrosome integrity percentages between treatments after arcsine transformation. In vitro fertilization data were analyzed by a generalized linear model (GLM) and a Tukey post hoc test was used to assess difference between treaments.

3. Results

3.1 Experiment 1: Effect of Res supplementation to thawed boar sperm on viability, acrosome integrity and IVF parameters

The addition of Res 0.5, 1 or 2 mM to thawed sperm for 1 h (Res 0.5, Res 1, Res 2) did not induce any significant effect on sperm viability as compared to semen without Res (CTR) (51.7% ± 9.4%, 48.5% ± 5.3, 48.4% ± 8.6, 43.8% ± 10.7 in CTR, Res 0.5, Res 1, Res 2 respectively) (Fig.1). Moreover, the supplementation of different concentrations of Res to thawed sperm for 1 h did not improve the percentage of spermatozoa with intact acrosome compared to CTR group (86.4 % ± 6.1 %, 82.5% ± 3.2 %, 81.9 % ± 2.6 %, 82.4% ± 1.0 %, in CTR, Res 0.5, Res 1, Res 2 respectively) (Fig. 1).

When Res 2 mM treated samples were used for IVF a significantly ($P<0.01$) higher penetration rate (number of oocytes penetrated/total inseminated) compared with CTR was observed (Table 1). Res 2 mM also exerted a positive effect ($P<0.01$) on the total efficiency of fertilization as compared to CTR group but did not induce any effect on monospermy rate (Table 1).

Table 1. Effect of Res (0.5, 1 and 2 mM) supplementation to thawed boar sperm on IVF parameters.

Group	N oocytes	Penetration rate %	Monospermy rate %	Total efficiency of fertilization
CTR	237	33.8 ± 12.4[a]	87.3 ± 5.5	29.6 ± 11.3[a]
Res 0.5	152	42.3 ± 2.7 [a]	73.2 ± 11.7	30.9 ± 5.1 [a]
Res 1	263	46.8 ± 5.0 [a §]	82.3 ± 12.5	38.5 ± 6.8 [a]
Res 2	192	68.8 ± 6.4 [b]	74.4 ± 12.3	51.0 ± 7.6 [b]

Penetration rate (number of fertilized oocytes / number of inseminated oocytes).

Monospermy rate (number of oocytes containing only one sperm head–male pronucleus / number of penetrated oocytes).

Total efficiency of fertilization (number of monospermic oocytes / number of inseminated oocytes).

Values are expressed as the mean ± SD of six replicates (three boars).

Different letters indicate significant difference for $P < 0.01$ in column between treatments.

§ indicates significant difference in column for $P = 0.05$ compared with control.

3.2 Experiment 2: Effect of EGCG supplementation to thawed boar sperm on viability, acrosome integrity and IVF parameters

The addition of different concentrations of EGCG (25, 50 and 100 μM) to thawed sperm for 1 h did not exert any significant effect on sperm viability (45.1% ± 3.8 %, 46.6 % ± 7.0 %, 45.4 % ± 10.1 %, 48.2 % ± 5.8 %, in CTR, EGCG 25, EGCG 50, EGCG 100 μM respectively) and acrosome integrity (86.2 % ± 5.5 %, 84.3% ± 6.3 %, 87.1 % ± 1.5 %, 85.4 % ± 1.5 %, in CTR, EGCG 25, EGCG 50, EGCG 100 μM respectively) (Fig. 1). Oocytes inseminated with thawed spermatozoa pretreated with all the different EGCG concentrations tested presented a significantly ($P < 0.01$) increased penetration rate compared to CTR (Table 2). In addition, 25 and 50 μM EGCG supplementation exerted a positive effect ($P<0.01$) on the total efficiency of fertilization without inducing any effect on monospermy rate (Table 2).

Table 2. Effects of EGCG (25, 5 and 100 μM) supplementation to thawed boar sperm on IVF parameters.

Group	N oocytes	Penetration rate %	Monospermy rate %	Total efficiency of fertilization
CTR	247	31.9 ± 9.4[a]	83.7 ± 8.8	26.8 ± 9.5 [aA]
EGCG 25	158	55.0 ± 5.3[b]	74.5 ± 19.5	40.3 ± 8.2 [aB]
EGCG 50	234	54.8 ± 9.0 [b]	74.5 ± 12.3	40.4 ± 7.8 [bB]
EGCG 100	244	48.6 ± 10.4 [b]	72.6 ± 18.7	34.6 ± 9.9 [aA]

Penetration rate (number of penetrated oocytes / number of inseminated oocytes).

Monospermy rate (number of oocytes containing only one sperm head–male pronucleus / number of penetrated oocytes).

Total efficiency of fertilization (number of monospermic oocytes / number of inseminated oocytes).

Values are expressed as the mean ± SD of six replicates (three boars).

Different lowercase letters indicate significant difference for $P < 0.01$ in column between treatments.

Different capital letters indicate significant difference for $P < 0.05$ in column between treatments.

4. Discussion

Sperm cryopreservation is the best technology to store boar semen for long periods for planning artificial insemination and preserving genetic material through time; nevertheless, frozen-thawed sperm is not routinely used in pig industry (less than 1%) [34]. The main reason is that freezing and thawing procedures lead to a reduced sperm fertilizing ability and reproductive performance [3] because of two important events occurring during cryopreservation procedure: the vast production of ROS and the parallel decrease in antioxidants defenses [35]. On this basis, in order to improve post-thaw quality of boar sperm, various antioxidants are routinely added during freezing protocols and new molecules are continuously studied [36].

In our study, Res supplementation did not induce any effect on sperm viability as already reported by other Authors even if in different experimental conditions and species: liquid storage of boar [37] and stallion [38] semen or cryopreservation of bull sperm [22].

Thawed boar spermatozoa can show membrane rearrangements and consequent lipid packing faults [39]. In this way, efficiency of calcium channels could be compromised, leading to an increase in calcium concentration inside the cell that could lead to capacitation like changes [40]. Liu et al. [41] reported that Res affects intracellular calcium release, so it could be important in preventing premature sperm capacitation and, consequently, acrosome reaction; in this study, however, Res supplementation to thawing media for one hour did not preserve sperm acrosome integrity. Similar results have been obtained by Martín-Hidalgo et al. [37] who reported that storage at 17°C of fresh boar semen with Res did not exert any effect on this parameter and by Silva et al. [23] who added Res to ram cryopreserved semen.

In our study, no protective effect of EGCG was observed on acrosome integrity in agreement with the results obtained by Vallorani et al. [29] on liquid storage of boar sexed semen. Different results have been shown by Kitaji et al. [28] who observed a higher post-thaw viability and acrosome integrity of boar spermatozoa incubated prior to freezing in a semen extender supplemented with 0.01% of green tea polyphenol extracts.

When either Res 2 mM or EGCG 25, 50 and 100 µM treated samples were used for IVF we observed a significantly higher penetration rate ($P<0.01$) compared with control; in addition, Res 2 mM and EGCG 25 and 50 µM supplementation exerted a positive effect ($P<0.01$, $P< 0.05$ and $P<0.01$ respectively) on total efficiency of fertilization.

Our results agree well with those from several studies examining the effect of EGCG during IVF in different experimental conditions. Pre-incubation of boar spermatozoa with green tea polyphenol extracts prior to freezing has been shown to increase both the efficiency of IVF (rates of monospermic

oocyte) and blastocyst formation [28]. Kaedei et al. [42] demonstrated that penetration rate improves when boar frozen-thawed spermatozoa are co-incubated with oocyte in IVF medium supplemented with 50 µM EGCG. A positive influence of EGCG has also been recorded on fresh boar and stallion spermatozoa under capacitating conditions, in which a significant increase in the number of sperm bound to oocyte zona pellucida was observed [43,44]. Moreover, the presence of this polyphenol during pig IVF using fresh semen was able to increase, in a dose response manner, the fertilization rate [43].

Therefore, EGCG could likely modulate sperm capacitation process probably thanks to its antioxidant activity; in fact, under capacitating conditions EGCG has been demonstrated to reduce H_2O_2 production in boar spermatozoa [43] and to be able, in stallion spermatozoa, to reverse the inhibition of mitochondrial complex I by rotenone, a molecule known to induce mitochondrial ROS production [44].

To our knowledge, Res treated semen has never been used for IVF so far, while it is known that Res supplementation during IVM and IVC improves developmental potential of porcine oocytes and porcine embryo development [45–47].

Studies performed on mouse, human, bovine and ram spermatozoa demonstrated that Res could effectively protect spermatozoa from oxidative stress induced by cryopreservation or pro-oxidant agents supplementation [17,19–23]. In our work, although Res 2 mM added to thawing sperm media for 1 hour did not exert any effect on parameters assessed (viability and acrosome integrity), it significantly ($P<0.01$) increased the penetration rate and total efficiency of fertilization. The encouraging beneficial effect of Res and EGCG addition to the thawing extender was evident during IVF and therefore after washing away the tested molecules: the two molecules were left with semen for 1 hour after thawing and then the medium was discarded and spermatozoa washed with fresh Bracket and Oliphant's medium. This suggests that the protective action during thawing can lead to positive effects on sperm function that, in turn, are responsible for the subsequent increased fertilizing ability even if the molecules are no more present. Therefore, it cannot be excluded (and it should certainly be tested) that Res or EGCG pretreatment of thawed semen could lead to positive effects also in vivo.

In conclusion, our results indicate that the addition of Res 2 mM or EGCG 25, 50 and 100 µM to thawed spermatozoa for one hour, even if does not exert any effect on sperm viability and acrosome integrity, improves in vitro penetration rate; in addition, EGCG 50 µM and Res 2 mM increases the total efficiency of fertilization. These results could be possibly important not only *in vitro*, but also *in vivo* as the addition of one of these two antioxidants in the commercial thawing solution might

enhance sperm fertilizing ability and reproductive performance during porcine AI with frozen-thawed boar semen.

Acknowledgements

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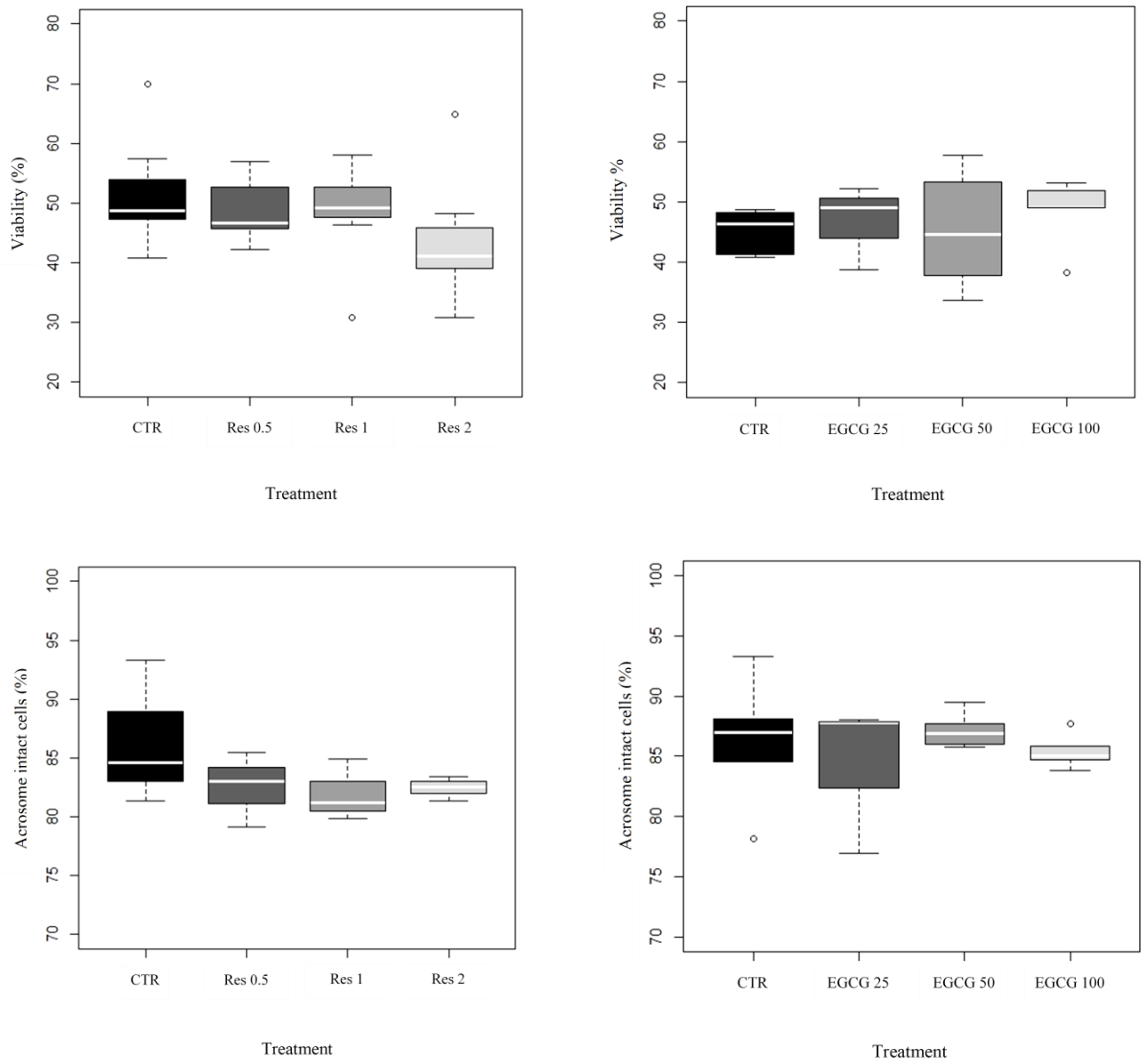
383 **Figure 1**

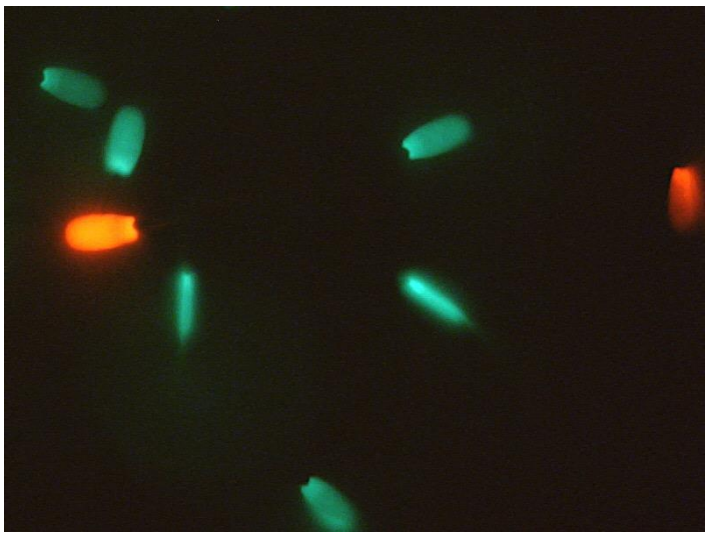
384



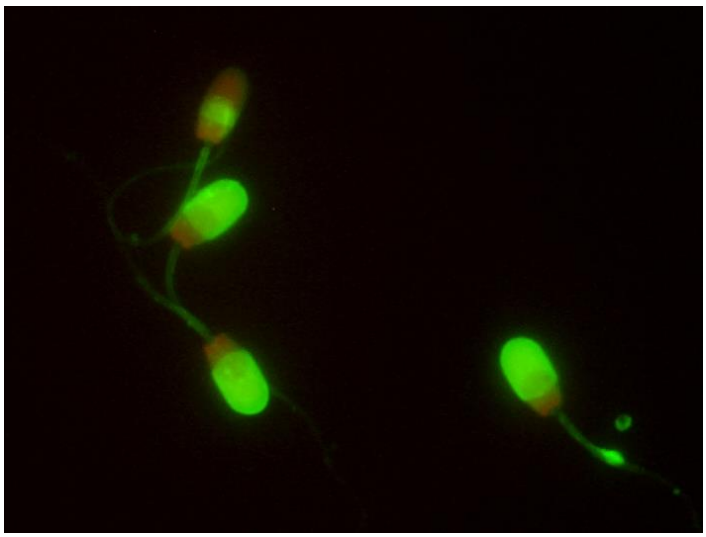
385

386 Fig. 1 Boxplots representing sperm viability (upper panels) and acrosome integrity (lower panels) of
387 frozen-thawed spermatozoa after 1 h of incubation at 37°C without (CTR) or with Res (0.5, 1 or 2
388 mM) (left panels) and EGCG (25, 50 or 100 µM) (right panel). Each experiment was repeated six
389 times (three boars).

Supplementary file



A. Representative fluorescent micrographs of semen stained with LIVE/DEAD® Sperm Viability kit (Molecular Probes, Invitrogen). The spermatozoa with green or red fluorescence on the head are considered live or dead, respectively.



B. Representative fluorescent micrographs of semen stained with FITC conjugated lectin from Pisum Sativum (FITC-PSA) and counterstained with Propidium Iodide. The presence of a green acrosomal fluorescence is indicative of an intact acrosome, while a partial or total absence of fluorescence indicative of acrosome disruption or acrosome reaction.

PAPER 2

"Combined effects of Resveratrol and Epigallocatechin-3-gallate on post thaw boar sperm and IVF parameters"

NOTICE:

This is the author's version of the manuscript under second revision in Theriogenology.

Title

Combined effects of Resveratrol and Epigallocatechin-3-gallate on post thaw boar sperm and IVF parameters

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Abstract

Frozen-thawed boar semen suffer a fertility decrease that negatively affects its widespread use. In recent years supplementing frozen-thawed boar sperm with different antioxidants gave interesting and promising results; the aim of the present work was to study the effect of supplementing boar sperm thawing medium with combination of Epigallocatechin-3-gallate (EGCG, 50 µM) and Resveratrol (R, 2mM), on boar sperm motility (assessed by CASA), viability, acrosome integrity, mitochondrial function, lipid peroxidation and DNA integrity (assessed by flow cytometry), protein tyrosine phosphorylation (assessed by immunofluorescence) and on in vitro fertilization (IVF).

After thawing, sperm cells were incubated 1 h at 37°C in BTS in absence or presence of R and EGCG, alone or in combination; after incubation, an aliquot of sperm suspension was used for sperm analysis (performed immediately and for a further 3 h incubation), another one was capacitated for 1 h at 39°C 5%CO_2 in Brackett and Oliphant's medium for immunolocalization of tyrosine-phosphorylated proteins, while the latter aliquot was used for IVF trials.

Our results demonstrate that sperm motility is negatively affected by R (alone or associated with EGCG) in comparison to control and EGCG groups both at 1h and 4 h; this effect is evident both in average motility parameters and in single cells kinematics (studied by cluster analysis).

Viability, acrosome integrity, mitochondrial functionality and lipid peroxidation are not influenced by the addition of the antioxidants; finally, DNA integrity is negatively influenced by R (both alone or associated with EGCG) both at 1h and 4h incubation. Finally, tyrosine phosphorylated protein immunolocalization, used as capacitation parameter, is not affected by the different treatments.

Penetration rate is strongly enhanced by R, both alone or associated with EGCG; EGCG increases penetration rate as well but to a lower extent.

Our findings demonstrate that the combination of R and EGCG could positively affect frozen-thawed boar sperm fertility in vitro; the effect is evident also in R groups, thus demonstrating that this antioxidant is predominant and no synergic effect is present. Some insights are needed to understand if, in particular R (that showed the strongest effect) could be profitably used for artificial insemination in vivo, given the detrimental effect of this molecule on both sperm motility and DNA integrity.

Keywords

Resveratrol
Epigallocatechin-3-gallate
Pig
Frozen-thawed spermatozoa

1. Introduction

Cryopreservation is a useful technique to store germinal cells, and in particular spermatozoa; in boar sperm production, this particular field represent an important challenge for commercial farm or AI centres as nowadays is not yet suitable for a widespread use and it is of utmost importance in gene banking, research and advanced reproductive biotechnologies [1,2].

During cryopreservation, sperm cells undergo some changes which are mainly concentrated in the cooling-freezing passage [3]. Sperm membrane is particularly susceptible to cryodamage, as in pig the cholesterol:lipid ratio is lower than in other species [4–6], moreover freeze-thawing can affect sperm nuclear proteins (histones and protamine) and DNA [7–9], and can lower mitochondrial activity [10]. Cryodamage also induces structural modifications in several proteins, such as membrane transporters (e.g. Ca^{2+} channels, glucose transporters) [10–12], impairs sperm motility and affects the distribution of the different motile subpopulations [13,14].

Attention has been given to damages derived from reactive oxygen species (ROS). These molecules have a recognized and important role in sperm function, as they are involved in sperm activation and capacitation [15–18]. These substances (e.g. anion superoxide, hydrogen peroxide and nitric oxide), when produced at controlled rates have a stimulatory role on some events related to capacitation, sperm-oocyte interaction and acrosome reaction [15,17], via the activation of some internal pathways (cyclic AMP-Protein Kinase A; mitogen activated protein kinase/extracellular regulated Kinases; protein tyrosine phosphorylation) that are also linked to hyperactivated motility and acrosome reactivity. However, the excessive accumulation of some of these molecules into the cell or in the surrounding environment may represent a danger for sperm cells [16,19–21]. In fact, and as previously reported [1,21], ROS can affect sperm integrity (i.e. sperm plasma membrane, acrosome, DNA, mitochondria), thereby impairing their function (motility, hyperactivation, sperm-oocyte interaction). Specific features of boar sperm cells are potential targets for ROS such as: polyunsaturated fatty acid (PUFA) and phospholipase A present in the membrane [21].

Several antioxidants (L-cysteine, α- tocopherol, lutein, butylated hydroxytoluene, Trolox, ascorbic acid, epigallocatechin-3-gallate, reduced glutathione, resveratrol) have been added to preservation

media to mitigate the adverse effects of ROS and cryopreservation in boar spermatozoa and to better maintain the sperm function [1,22–24]. While, some molecules, such as ascorbic acid and reduced glutathione, have been demonstrated to be active and powerful, either alone or combined [24,25], others, such as resveratrol , have not been found to exert a positive impact [26]. The latter, anyway, showed an interesting effect on vitrified porcine oocytes [27] and may be much efficient in protecting from the freezing process.

In a recent study, we supplemented the thawing medium with natural antioxidants (Epigallocatechin-3-gallate and Resveratrol) and observed an increase in vitro fertilization rate of boar frozen semen and no change in either sperm viability and acrosome integrity [22].

Based on the above-mentioned researches the aim of the present study was to test the effects of the combination of Resveratrol (R) and Epigallocatechin-3-gallate (EGCG) added to thawing medium on both sperm parameters and fertilizing ability.

2. Materials and Methods

Unless otherwise specified, all chemicals were purchased from Sigma-Aldrich (Saint-Louis, MO, USA).

2.1 Experimental design

Boar semen frozen in 0.5 mL straws was purchased from a commercial company (Inseme S.P.A., Modena, Italy).

Three straws from the same ejaculate were thawed in a water bath at 37°C under agitation for 30 seconds and subsequently pooled and diluted in Beltsville Thawing Solution (BTS) at a dilution rate 1:3. Only thawed samples with sperm viability higher than 40%, as evaluated by SYBR14/PI test (see below), were used. Thereafter, each sample was divided into four aliquots, each corresponding to one of the following treatments: control (CTR), Resveratrol 2 mM (R), Epigallocatechin-3-gallate 50 µM (EGCG) and R+EGCG at the same concentrations.

The antioxidants doses were chosen on the basis of the results of our previous work [22].

Sperm suspensions were kept for 1 h at 37°C in the dark and subsequently used in Experiment 1 or 2.

2.1.1 <u>Experiment 1</u>. Assessment of sperm parameters

An aliquot (500 µL) of each sperm suspension incubated at 37°C for 1 h in the dark was used to evaluate: sperm motility, viability, acrosome integrity, mitochondrial activity, lipid peroxidation and

DNA integrity (See below for description of the methods). The remaining part of sperm suspension was kept at 37°C for an additional 3-h period in the dark and the same analyses were subsequently performed as reported by other Authors [28]. Four different animals (1 ejaculate each) were used and each experiment was repeated three times.

For tyrosine phosphorylation immunostaining analyses, an aliquot of spermatozoa incubated for 1 h in the dark at 37°C from each experimental group was fixed, while another aliquot was washed twice in Brackett and Oliphant's (BO) medium [29] supplemented with 12% foetal calf serum (FCS) and 0.7g/L caffeine (IVF medium) and then resuspended in the same medium at a final concentration of $30x10^6$ spermatozoa/mL and incubated 1 h under capacitating condition (5%CO_2, 39°C). Finally, samples were fixed for immunolocalization of tyrosine phosphorylated proteins. Three different animals (1 ejaculate each) were used and each experiment was repeated twice (n=6).

2.1.2 Experiment 2. Effects of R and EGCG on sperm fertilizing ability

After 1 h incubation in BTS with the different antioxidants, aliquots of sperm cells were used for in-vitro fertilization (IVF) trials and final sperm concentration was adjusted to $1x10^6$ spz/mL (See Section 2.4). Oocytes were treated as described in Section 2.4.

Three different animals (1 ejaculate each) were used and each experiment was repeated three times for IVF (n=9).

2.2 Sperm motility assessment

Sperm motility was assessed using a computer-assisted sperm analysis system (CASA, Hamilton Thorne, IVOS Ver. 12); the standard boar setup was used (60 frame per sec; 45 n. of frames; min contrast 49; min cell size 6 pixels; progressive cells: VAP 20.1 µm/sec; straightness percentage 75; static cell cutoff: VAP 20 µm/sec, VSL 5 µm/sec). Approximately one thousand cells at $30×10^6$ sperm/mL were evaluated for each sample using a fixed-height Leja Chamber SC 20-01-04-B (Leja, The Netherlands). Parameters assessed were percentages of total motile spermatozoa (TM), percentages of progressively motile spermatozoa (PM), curvilinear velocity (VCL µm/sec), average path velocity (VAP µm/sec), straight line velocity (VSL µm/sec), percentages of straightness (STR) and linearity (LIN), average lateral head displacement (ALH µm) and beat cross frequency (BCF Hz). Together with global sample analysis, individual sperm tracks were assessed and VCL, VAP, VSL, STR, LIN, ALH and BCF were recorded for each motile spermatozoon. These parameters were used to study the distribution of sperm subpopulations in all treatments (See statistical analysis – cluster analysis for sperm motion).

137 *2.3 Flow cytometry analysis*

138 Information about flow cytometry analyses is reported taking into account the recommendations of
139 the International Society for Advancement of Cytometry [30]. Flow cytometry analyses were
140 conducted to evaluate sperm viability, acrosome integrity, mitochondrial function, lipid peroxidation
141 levels and DNA fragmentation. In each assay, sperm concentration was adjusted to 1×10^6
142 spermatozoa/mL in a final volume of 0.5 mL BTS, and spermatozoa were then stained with the
143 appropriate combinations of fluorochromes, following the protocols described below. Samples were
144 evaluated through a FACSCalibur flow cytometer (Becton Dickinson, Milan, Italy) equipped with a
145 488 nm argon-ion laser. Emission measurements were made by means of three different filters:
146 530/30 band-pass (green/FL-1), 585/42 band-pass (orange/FL-2) and >670 long pass (far red/FL3)
147 filters. Data were acquired using the BD CellQuest Pro software (Becton Dickinson).
148 Signals were logarithmically amplified and photomultiplier settings were adjusted to each particular
149 staining method. FL1 was used to detect green fluorescence from SYBR14, fluorescein
150 isothiocyanate (FITC)-conjugated *Pisum sativum* agglutinin (PSA), low mitochondrial membrane
151 potential (JC1 negative), and BODIPY 581/591, whereas FL2 was used to detect orange fluorescence
152 from high mitochondrial membrane potential (JC1 positive) and FL3 was used to detect orange-red
153 fluorescence from propidium iodide (PI).
154 Side scatter height (SS-h) and forward scatter height (FS-h) were recorded in logarithmic mode (in
155 FS vs. SS dot plots) and sperm population was positively gated based on FS and SS while other events
156 were gated out. A minimum of 10,000 sperm events were evaluated per replicate.
157 In FITC-conjugated PSA flow cytometric assessment, percentages of non-DNA–containing particles
158 (alien particles), (*f*) were determined to avoid an overestimation of sperm particles in the first quadrant
159 (q_1) as described by [31], according to the following formula:

160 $$q'_1 = \frac{q_1 - f}{100 - f} \times 100$$

161 where q'_1 is the percentage of non-stained spermatozoa after correction.

162 *2.3.1. Sperm membrane integrity (SYBR14/PI)*

163 Sperm viability was assessed by checking the membrane integrity using two separate fluorochromes
164 SYBR-14 and PI (LIVE/DEAD Sperm Viability Kit; Molecular Probes, Invitrogen, Milan, Italy).
165 SYBR-14 is a membrane-permeable dye, which stains the head of viable spermatozoa in green, while
166 PI is a membrane-impermeable dye that only penetrates through disrupted plasma membrane, staining

the sperm heads of non-viable cells in red. Sperm samples were diluted with BTS to a concentration of 1×10^6 spermatozoa/mL and aliquots of 500 µL were stained with 5 µL SYBR-14 working solution (final concentration: 100 nM) and with 2.5 µL of PI (final concentration: 12 µM) for 10 min at 37°C in darkness. Viable spermatozoa exhibited a positive staining for SYBR-14 and negative staining for PI (SYBR-14+/PI-). Single-stained samples were used for setting the voltage gain for FL1 and FL3 photomultipliers.

2.3.2 Acrosome integrity analysis (PSA-FITC/PI)

Sperm acrosome intactness was assessed by Pisum sativum agglutinin (PSA) conjugated with fluorescein isothiocyanate (FITC) (2.5 mg/mL stock solution; 0.5 mg/mL working solution) coupled with Propidium Iodide (2.4 mM stock solution). Sperm samples were diluted with BTS to a concentration of $1 \cdot \times 10^6$ spermatozoa/mL and aliquots of 500 µL were stained with 10 µL FITC-PSA (final concentration: 10 µg/mL) and with 3 µL PI (final concentration: 14 µM) for 10 min at 37 °C in darkness. Four different sperm subpopulations were distinguished: a) viable acrosome-intact spermatozoa were those cells that did not stain with either FITC-PSA or PI and appeared in the lower left quadrant of FL1 *vs.* FL3 plots; b) viable spermatozoa with disrupted acrosome stained only in green with FITC-PSA and were found in the lower right panel; c) non-viable spermatozoa with intact acrosome stained with PI only and appeared in the upper left quadrant; and d) non-viable spermatozoa with disrupted acrosomes were found in the upper right quadrant and stained positively with both stains.

2.3.3 Mitochondrial membrane potential analysis (JC1/PI)

5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolyl carbocyanine iodide (JC-1) was used to evaluate mitochondrial membrane potential. When it comes in contact with mitochondria with high membrane potential, JC-1 forms multimers (known as J-aggregates) and emits orange fluorescence at 590 nm, which is detected by FL-2 photomultiplier. In contrast, when mitochondria have low membrane potential, JC-1 maintains its monomeric form (M-band) and emits green fluorescence at 530 nm, which is detected by FL-1 photomultiplier.

Sperm samples were diluted with BTS to a concentration of $1 \cdot \times 10^6$ spermatozoa/mL and aliquots of 500 µL were stained with 5 µL JC1 (at a final concentration of 1µg/mL) and 3 µL of PI (at a final concentration of 14 µM); samples were successively incubated at 37 °C for 30 min in the dark.

PI positive cells were gated out in a FL-1/FL-3 dot plot; PI negative cells were gated and analysed in a FL-1/FL-2 plot. High mitochondrial membrane potential cells (HMMP) stained orange (higher FL-2) and low mitochondrial membrane potential cells (LMMP) stained green (higher FL-1).

2.3.4 Lipid peroxidation analysis (Bodipy/PI)

BODIPY 581/591 (Molecular Probes Eugene, CA, USA) stock solution was prepared diluting 1 mg of the molecule in 1980 µL DMSO. For analysis, sperm samples were diluted with BTS to a concentration of $1 \cdot \times 10^6$ spermatozoa/mL; aliquots of 500 µL were centrifuged at 900x g for 2 min at room temperature; the supernatant was discarded, and sperm pellet resuspended with 492 µL BTS and stained with 5 µL BODIPY stock solution (final concentration 0.01 ug/mL and 3 µL of PI (at a final concentration of 14 µM). Cells were incubated for 30 min at 37 °C in the darkness and subsequently analysed.

As no separate sub-populations in FL1-FL3 plots were detectable, a relative fluorescence quantification method was used, as described by [19]. Briefly, the instrument was set with 10 references of the same ejaculate of frozen-thawed boar semen and the mean FL1 signal was registered. For each analysis, one sample of the same reference was used to set the voltage and gain of the instrument to get the same reference value; subsequently the experimental samples were run.

2.3.5 Sperm chromatin structure assay (SCSA)

Sample preparation and processing, as well as flow cytometer adjustments, were performed as previously described [32–34]. Briefly, 50 µl of each semen sample were immediately frozen and stored at −80°C until analysis (maximum 2 weeks). Sperm samples were handled individually and were thawed in a 37°C water bath. Immediately after thawing (30–60 s), aliquots of thawed semen were added with 200 µL of a buffer solution (0.186 g disodium EDTA, 0.790 g Tris–HCl and 4.380 g NaCl in 500 mL deionized water; pH adjusted to 7.4). This was mixed with 400 µL of an acid detergent solution (2.19 g NaCl, 1.0 ml of 2 N HCl solution, 0.25 ml Triton X, and deionized water quantum sufficit to a final volume of 250 ml). After 30 sec, 1.2 ml of the acridine orange solution were added Cell flow rate was set on low which resulted in an actual flow rate of 100–200 cells/sec. A total of 5,000 events was evaluated for each sample. Sperm from a single control boar were used as a biologic control to standardize instrument settings between days of use. The flow cytometer was adjusted such that the mean green fluorescence was set at the 500 channel (FL-l at 500) and mean red fluorescence at the 150 channel (FL-3 at 150). Data were acquired in a list mode (linear scale), and

analysis was performed using winlist software (Verity Software House). The percentage of sperm with abnormal DNA was defined by the parameter DNA fragmentation index (DFI).

2.4 In vitro fertilization (IVF) trials

Oocytes were cultured as already described [22]; briefly, ovaries were obtained from pre-pubertal gilts at a local abattoir. Cumulus oocyte complexes (COCs) from follicles 3–6 mm in diameter were aspirated using a 18-gauge needle attached to a 10-mL disposable syringe. Intact COCs were selected under a stereomicroscope and only COCs with more than two layers of intact cumulus oophorus and with uniform cytoplasm were used. Next, COCs were washed three times with NCSU 37 [35] supplemented with 5.0 µg/mL insulin, 1mM glutamine, 0.57 mM cysteine, 10 ng/mL epidermal growth factor (EGF), 50 µM ß -mercaptoethanol and 10% porcine follicular fluid (IVM medium). Groups of 50 COCs were transferred to a Nunc 4-well multidish containing 500 µL of the same medium per well and in vitro matured at 39°C and 5% CO_2 in a humidified atmosphere. During the first 22 h of in vitro maturation, IVM medium was supplemented with 1.0 mM db-cAMP, 10 IU/mL equine chorionic gonadotropin (eCG) (Folligon; Intervet, Boxmeer, The Netherlands) and 10 IU/mL human chorionic gonadotropin (hCG) (Chorulon; Intervet). For the last 22–24 h of IVM, COCs were transferred to fresh maturation medium [36].

Groups of 50 matured oocytes, freed from cumulus cells by repeated gentle pipetting, were transferred to 500 µL IVF medium containing 1×10^6 spz/mL. After 1 h of co-culture, oocytes were transferred to fresh IVF medium and cultured for 20 h. The oocytes were then mounted on microscope slides, fixed in acetic acid/ethanol (1:3; v:v) for 24 h and stained with Lacmoid. Oocytes were observed under a phase-contrast microscope and parameters evaluated were: penetration rate (number of oocytes penetrated/total inseminated), monospermy rate (number of oocytes containing only one sperm head–male pronucleus/total fertilized) and total efficiency (number of oocytes containing only one sperm head–male pronucleus/total inseminated). Degenerated and immature oocytes were not counted.

2.5 Immunolocalization of tyrosine phosphorylated proteins in spermatozoa

Sperm cells preparation were analysed as described by [37]; sperm cells ($30x10^6$ spermatozoa/mL) were spread onto poly-L-lysine-coated slides, fixed with absolute methanol at -20°C for 10 min and then with acetone for 30 sec. Slides were washed with phosphate buffered saline solution (PBS), let dry and then blocked with 10% (v/v) FCS in PBS for 30 min. A primary antibody against tyrosine phosphorylated proteins (Upstate Millipore, Watford, UK) was added at a 1:200 dilution (v:v) in PBS

10% FCS. Incubation was carried out overnight at 4°C in humid chambers. After three washings in PBS, slides were incubated with a goat anti-mouse (dilution 1:800), FITC-conjugated secondary antibody for 1 h in the dark. Next, slides were washed again three times in PBS and mounted with anti-fading Vectashield mounting medium with PI (Vector Laboratories) to counterstain the nuclei. Negative controls were treated similarly with the omission of the primary antiserum. Images were obtained using a Nikon digital camera installed on a Nikon epifluorescence microscope (Nikon Inc., Melville, NY, USA). Two-hundred cells were counted for each replicate.

Different patterns of tyrosine phosphorylated sperm proteins were identified, following the description by [37] with some modifications: A: spermatozoa with acrosome and equatorial sub segment positivity; B: spermatozoa with tail positivity (whole tail or the principal piece only) and acrosome and/or equatorial sub-segment positivity; C: spermatozoa with tail positivity (whole tail or principal piece only); and NEG: spermatozoa with no positive signal.

2.6. Statistical analyses

Statistical analyses were performed using R (version 3.4.0). Values are expressed as mean ± standard deviation (SD), unless otherwise specified and level of significance was at $P \leq 0.05$.

2.6.1 Effects of treatment upon sperm function parameters, tyrosine phosphorylation and IVF outcomes

Motility and post thawing parameters assessed by flow cytometry expressed as percentages were transformed with arcsine square root. Subsequently all variables (both motility and post thawing parameters) were tested for normality and homogeneity of variances through Shapiro-Wilk and Levene tests. Then, a mixed effect model was set to determine treatment and time effects (1 and 4 h at post-thaw) and their interaction, with the boar ejaculate and repetition as random effects.

As for IVF trials, the variables (i.e. penetration rates and monospermy) were analysed using a general linear model with binomial distribution and a Tukey post-hoc test was subsequently run to determine differences between treatments.

Finally, tyrosine phosphorylation data were arcsin square root-transformed. Normality of the residues was checked by Shapiro Wilk test and homogeneity of the variances was tested with Levene test. One-way ANOVA and Tukey post hoc test were used to assess differences between treatments.

285 *2.6.2 Cluster analysis for motility parameters*

286 Sperm kinematics parameters were registered for each individual motile sperm cell from three boars,
287 each analysed twice at 1 and 4 h post-thaw; ALH, BCF, VCL, VAP, VSL, LIN and STR were
288 recorded and used for cluster analysis.
289 As reported by [38], data were first normalized, then a principal component analysis was performed
290 to reduce the total number variables. Finally, a hierarchical clustering using the Ward's method and
291 Euclidean distances was applied to the Principal components and 4 clusters were identified. Finally,
292 a chi square analysis was used to assess difference in the composition of the different clusters in
293 relation to treatment. In total, 17,953 cells were analysed.

294 **3. Results**

295 *3.1. Sperm motility*

296 *3.1.1 Sperm kinematics*

297 EGCG did not affect sperm motility in comparison with CTR, while R and R+EGCG affected all
298 sperm parameters. In effect, TM, VAP and VCL in R and R+EGCG treatments were significantly
299 ($P<0.05$) lower than CTR and EGCG at both 1 and 4 h post-thaw. In addition, the overall motility
300 parameters were found to decrease throughout post-thaw incubation time (i.e. 1 h *vs.* 4 h post-thaw;
301 Supplementary file 1 and Fig.1).
302 On the other hand, whereas PM, VSL, BCF, LIN and STR were also significantly lower in R and
303 R+EGCG treatments than in CTR and EGCG, the effects of post-thaw incubation time were less
304 apparent (Supplementary file 1 and Fig.1). Finally, ALH showed a significant decrease due to
305 treatment (with resveratrol) and post-thaw incubation time and their interaction (Supplementary
306 file1).

307 *3.1.2 Cluster analysis of motility parameters*

308 The principal component analysis resulted in four principal components that were used for subsequent
309 cluster analysis (Supplementary file 2). The resulting analysis showed four distinct clusters. These
310 different clusters showed different motion characteristics (Table 1) that allowed us to identify them
311 as: 1) slow non-progressive; 2) average; 3) rapid progressive; 4) rapid non-progressive.

Spermatozoa were assigned to the different clusters and, as reported in Table 2, significant differences in the percentages of spermatozoa belonging to each subpopulation were found between treatments (CTR, R, EGCG, R+EGCG).

3.2 Flow cytometry parameters

Sperm viability, evaluated through SYBR14/PI test, was not influenced by treatments, but a significant reduction in this parameter was observed in all the treatments throughout post-thaw incubation time (1 and 4 h of incubation after thawing) (Fig. 2, panel A).

Percentages of viable spermatozoa with an intact acrosome, evaluated by PSA-FITC/PI test, were not influenced by treatment or post-thaw incubation time (Fig. 2, panel B).

With regard to mitochondrial membrane potential, and as shown in Fig 3, panel A, percentages of viable spermatozoa displaying high mitochondrial membrane potential were not influenced by the treatment. However, those percentages were significantly reduced throughout post-thaw incubation time.

Lipid peroxidation measurement showed no significant differences between treatments and times of post-thaw incubation (Fig. 3, panel B).

Finally, sperm DNA integrity, measured by DNA fragmentation index (SCSA), showed significant differences between treatments (R and R+EGCG compared to CTR and EGCG) and for R and R+EGCG groups, times of incubation (Fig. 4)

3.3 Immunodetection of tyrosine phosphorylation patterns in sperm cells

After 1 h incubation in BTS, all experimental groups (CTR, EGCG, R, EGCG+R) showed the same pattern, with around 95% showing A-pattern and the remaining 5% showing no positivity. Although the percentage of cells showing the different patterns changed after 1 h of incubation in capacitating conditions (incubation in IVF medium for 1h at 39° C in a humified chamber 5% CO_2), no significant effect between treatments was observed (Table 3).

3.4 IVF trials

Penetration rates were found to be increased when R and/or EGCG were added. Notwithstanding, the extent of that increase compared to control was even higher when both R and EGCG (R+EGCG) were added in combination ($P<0.001$; Fig.5, panel A). Monospermy (number of oocytes penetrated by only one spermatozoon divided by the total number of fertilized oocytes) was also affected by

341 treatments, as R and R+EGCG exhibited significantly ($P<0.05$) lower monospermy rates than CTR
342 and EGCG (Fig.5, panel B).
343 Total efficiency was not influenced by the different treatments (Fig.5, panel C).
344 The total number of oocytes analysed was 1594, divided into the different groups as follow: 395
345 oocytes CTR, 435 EGCG, 378 R, 386 R+EGCG.

346 **4. Discussion**

347 Cryopreservation induces some alterations on boar spermatozoa that bring about a loss of fertility
348 [39], due to numerous effects strictly related to the process [1,2]. One concern is the excessive
349 generation of ROS that tends to induce hyper-oxidation damage in various sperm structures, such as
350 plasma membrane, DNA, nucleus and mitochondria [17,20,40].
351 The present work aimed at limiting the negative effects of ROS generation by adding, in combination,
352 two different natural antioxidants, R and EGCG after thawing boar spermatozoa. A previous study
353 [22], demonstrated that 50 µM EGCG and 2 mM R were the most effective doses and resulted in the
354 highest IVF rates of *in vitro* matured oocytes following IVF [22]. The same effect was observed also
355 in the present work. However, the most noticeable finding of this work after evaluating the single and
356 combined effects of both antioxidants, was that R is more powerful than EGCG in enhancing
357 penetration rates in vitro. In fact, whereas ECGC induced a significant increase in the percentage of
358 fertilized eggs when compared to the control, the extent of that increase was even higher in the case
359 of R. However, no synergic effect was observed when the two antioxidants (R+EGCG) were
360 supplemented in combination. The effects on fertilization were promising: the increase in penetration
361 rates could be very encouraging for the addition of these substances to post-thawing media for boar
362 spermatozoa, being that post-thawing fertility seems to be one of the major limitations for the use of
363 frozen-thawed boar semen in a wide scale [39,41]. In this regard, artificial insemination trials should
364 be performed to verify whether the strong effect observed in IVF is maintained *in vivo*, which would
365 involve an effect on both farrowing rates and litter sizes. The selection of "good freezing" boars is a
366 central strategy for the application of frozen-thawed boar semen on a large scale; the males we used
367 for IVF trials were chosen because of their good semen quality after thawing and their ability to
368 fertilize oocytes *in vitro*.
369 One of the main questions raised from these results is which mechanism could explain the strong
370 effect on IVF.
371 The first trial we performed, together with IVF, was aimed to study the localization of tyrosine
372 phosphorylated proteins in sperm cell; as we did in other reports [37,42]. This parameter, known to

be related to sperm capacitation, was used to detect different sperm subpopulations patterns (A, B, C and Neg) [37] and we studied the changes in their distribution due to the aforementioned treatments. Immunolocalization results clearly showed no difference between groups. Evidently, this parameter could be retained too "downstream" in the capacitation events to be thoroughly changed by the different treatments [37]. It should also be remarked that, after 1 h of incubation in BTS medium, no difference was found between groups; in particular, almost 95% of the sperm cells showed the A pattern, typical of non-capacitated spermatozoa.

To better determine whether any change on sperm metabolism and membrane composition resulted from the addition of R and ECGC to frozen-thawed spermatozoa, we studied a wider panel of sperm parameters, while only viability and acrosome integrity were evaluated in our previous work [22]. We assessed sperm viability, acrosome integrity, mitochondrial membrane potential, lipid peroxidation and DNA integrity through flow cytometry and sperm motility (including motile subpopulations) by CASA. All the flow cytometric analyses were performed both at 1 and 4 h after thawing, keeping the semen at 37°C.

Sperm viability and acrosome integrity were not influenced by the addition of antioxidants. These results match with those obtained in our previous study [22] even if the current study evaluated those parameters by flow cytometry and our previous work used epifluorescence microscope. It is worth noting that there was a decrease in viable spermatozoa throughout post-thaw incubation time (i.e. between 1 h and 4 h of incubation).

Regarding the metabolic status of spermatozoa, we checked mitochondria functionality of viable cells, with a particular emphasis on the percentages of viable spermatozoa with high mitochondrial membrane potential (HMMP) as reported by [43]. These percentages did not change between treatments thus indicating that these two natural antioxidants have no impact on mitochondrial function. In addition, a significant, even if not dramatic, drop in HMMP cells was recorded between 1 and 4 h of incubation. In this context, one should note that the control semen after 1 h of incubation exhibited a very high percentage of viable cells with HMMP (around 85%), and it would therefore have been difficult to further increase this percentage. Other reports indicate that mitochondrial function of boar spermatozoa during and after cryopreservation is impaired [10]; however, no distinction between viable and non-viable spermatozoa was made by the aforementioned work, which could explain why their data were different compared to ours.

From our results, it is reasonable to affirm that those cells that survive to cryopreservation are effectively functionally intact, both after 1 and 4 h of incubation at 37°C. As natural antioxidants were not effective in increasing the high percentage of cells with HMMP, the key point in ameliorating freezing protocols is to maintain as many viable spermatozoa as possible after freeze-

thawing procedures. It should be also taken into account that mitochondrial activity in boar spermatozoa does not seem to be impaired by ROS (that could be formed in excess during the cryopreservation process) [44]. This information is supported by other reports [8,45–48] that clearly show that the main energy source for boar spermatozoa is not the oxidative phosphorylation (taking place in the mitochondria), but anaerobic glycolysis [47,49].

Lipid peroxidation was investigated through Bodipy; also, for this parameter we did not find any difference between treatments and no changes between 1 and 4 h of incubation were observed. We checked the mean fluorescent intensity of Bodipy fluorochrome exhibited by viable spermatozoa and it was evident that viable spermatozoa surviving to cryopreservation did not undergo a dramatic change in lipid peroxidation. These data confirm that the oxidative process after cryopreservation is not so heavily detrimental for porcine sperm cells [23,50] and, thus, that impact of the addition of antioxidants may only be marginal.

Together with the high positive impact of R and EGCG on IVF, a very strong effect of R is evident on sperm motility: both total and progressive motility were negatively affected by R. Also, the other motility parameters followed the same trend, with a detrimental effect caused by R. The reason for this drop in motility is not clear. Whereas some Authors showed a positive effect of R on rat, bull and human sperm motility [51–53] during liquid storage, other reports on equine [54] and porcine species [26] indicated that this molecule exerts detrimental effects on sperm motility. However, cryopreserved sperm cells seem to be more sensitive to this antioxidant. The key point is that, although spermatozoa motility (checked at 1 h incubation at 37°C, at the beginning of the incubation time for IVF) is evidently compromised by R, their fertilizing ability was highest. This surprising and, to some extent, paradoxical effect deserves further research. Therefore, future studies should verify whether frozen-thawed boar sperm treated with R also exhibits higher reproductive performance *in vivo*. While the *in vitro* system is standardized and allows understanding some features of the fertilization process, there is great difference between in vivo and in vitro environment, the first one being more “selective” towards spermatozoa and the second one being fitted to obtain a good fertilization rate. In this context, another question that arises is whether R-treated sperm cells are able to pass the selection operated by the female genital tract and to fertilize a higher number of oocytes if compared to untreated frozen-thawed spermatozoa. At present, we have no data to answer this question, as no experimental evidence is provided from in vivo trials yet.

To better delineate the features of R-treated sperm motility, we performed cluster analysis using kinematics parameters of single sperm cells [13,55,56]. On the basis of cluster analysis sperm were classified into four clusters, with the following characteristics: one included sperm cells with low VSL, VCL, VAP and linearity and was considered as “slow non-progressive”; the second showed

441 average parameters' values; the third showed high velocity and highly linear cells (high VSL, LIN
442 and STR) and was considered to be "rapid progressive"; finally, the last one showed high velocities
443 but low linearity and was defined as "rapid non-progressive". The effects of R on the proportions of
444 each sperm subpopulation were very apparent, as those treatments in which R was present (R and
445 R+EGCG) showed a significant increase in the percentage of slow non-progressive cells, which was
446 concomitant with a dramatic decrease in the percentage of "rapid progressive" cells. In addition, at 1
447 h, there was a significant increase in the percentage of "rapid non-progressive" cells, more evident in
448 the R groups, but also present in the EGCG one, while at 4 h this cluster is equally represented in
449 both treatments. If we consider "rapid non-progressive" cells as hyperactivated-like spermatozoa
450 [57], this could provide a proof of the ameliorative effect of EGCG and particularly of R on IVF.
451 Therefore, under the controlled IVF environment and the small IVF volume of the incubation dish, it
452 is likely that a higher number of "hyperactivated" cells could reach the oocytes.
453 The last result deserving discussion regards DNA integrity. SCSA assay showed a noticeable effect
454 of R, as the presence of this antioxidant in the thawing medium significantly increased the percentage
455 of spermatozoa with fragmented DNA both at 1 and 4 h post-thaw. This fact was really surprising, as
456 other articles reported that freeze-thawing induces slight some detrimental changes on boar sperm
457 nucleus, in particular regarding the protamine-histone-DNA structure and the integrity of disulphide
458 bonds between nucleoproteins [7,41]. Other reports evaluated DNA fragmentation in boar
459 spermatozoa using SCSA and found no difference between fresh and frozen-thawed semen [58,59].
460 The levels DFI reported in those studies were similar to those obtained in our control group.
461 Therefore, our results on R effects should be interpreted taking into account that [59,60] found
462 negative correlations between DFI and farrow rate and average total number of pigs born, thus
463 suggesting that a spermatozoon with a fragmented DNA can fertilize an egg, but the outcome is lower
464 than that obtained with intact spermatozoa.

465 In conclusion, R and EGCG showed a positive effect on in vitro fertility of boar spermatozoa if added
466 after thawing, as they both increased penetration rate, with R being much influent on this parameter,
467 so that it masked the effect of EGCG when the two antioxidants were used together showing no
468 synergic effect. Anyway, R showed a negative impact on boar frozen thawed spermatozoa because it
469 negatively affects sperm motility and DNA integrity. All other parameters indicate that both the
470 molecules are, as other antioxidants, almost ineffective (viability, acrosome integrity, lipid
471 peroxidation, mitochondrial function). Taken together these results are difficult to be explained: from
472 one side boar sperm characteristics are poorly enhanced or negatively affected by the molecules, but
473 on the other side, a positive effect on sperm function is evident.

On the basis of our results it is necessary to understand if the positive effect is also maintained in vivo; in addition, further studies are needed to understand the effective mechanism by which the molecules act and to verify whether the development of the *in vitro* fertilized zygotes is normal or could be negatively affected.

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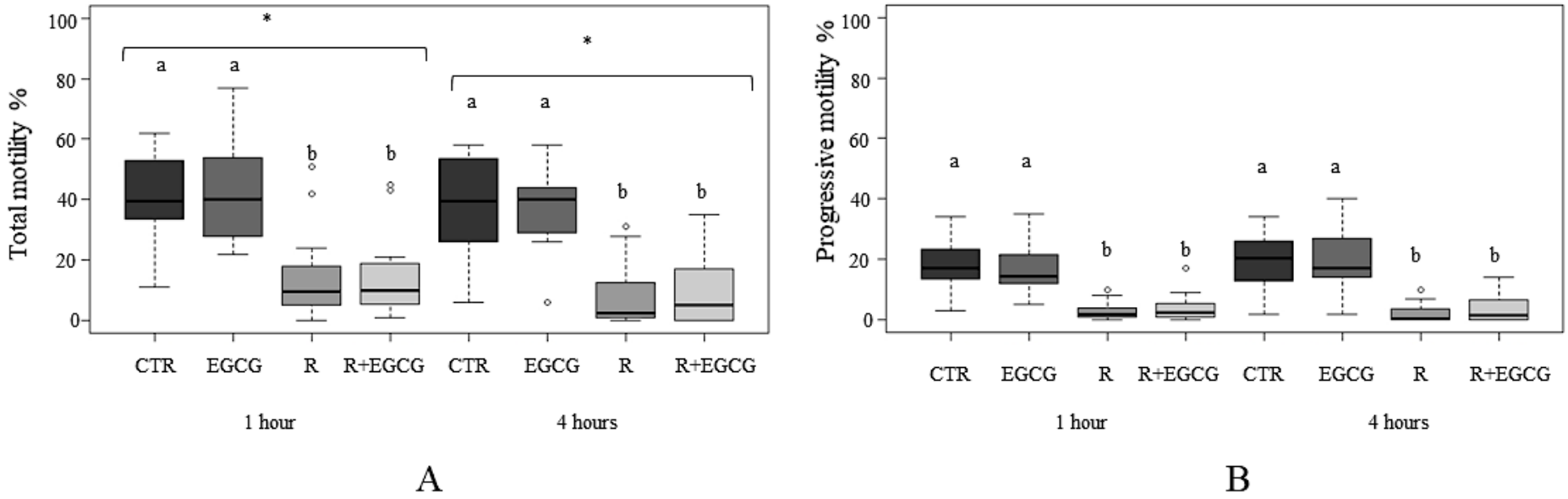
Figure 1

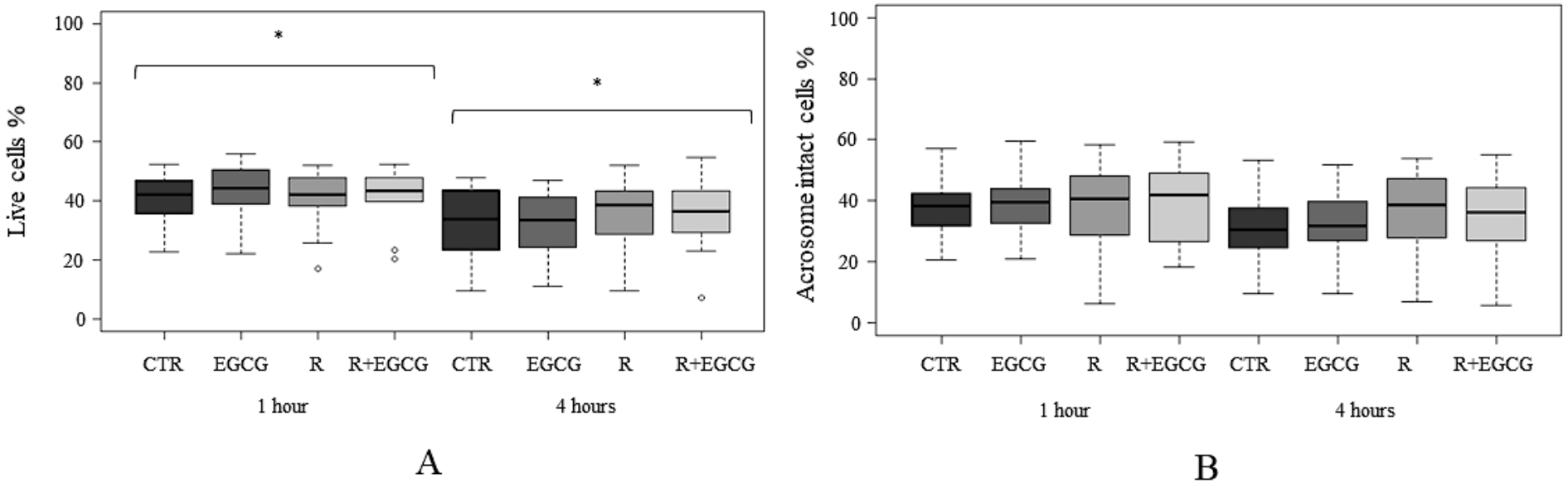
Figure 1. Percentage of motile (panel A) and progressively motile (panel B) frozen-thawed boar spermatozoa differently treated.



Horizontal line represents median; boxes represent 25-75 percentile; whiskers represent min-max, dots represent outliers. Different superscripts indicate significant difference for $p<0.05$; * indicates overall significant difference ($p<0.05$) between time 1 and time 4h; Four boars were used (one ejaculate each) with three replicates for each boar (n= 12). CTR: control; EGCG: Epigallocatechin-3-gallate 50 µM; R: Resveratrol 2µM.

Figure 2

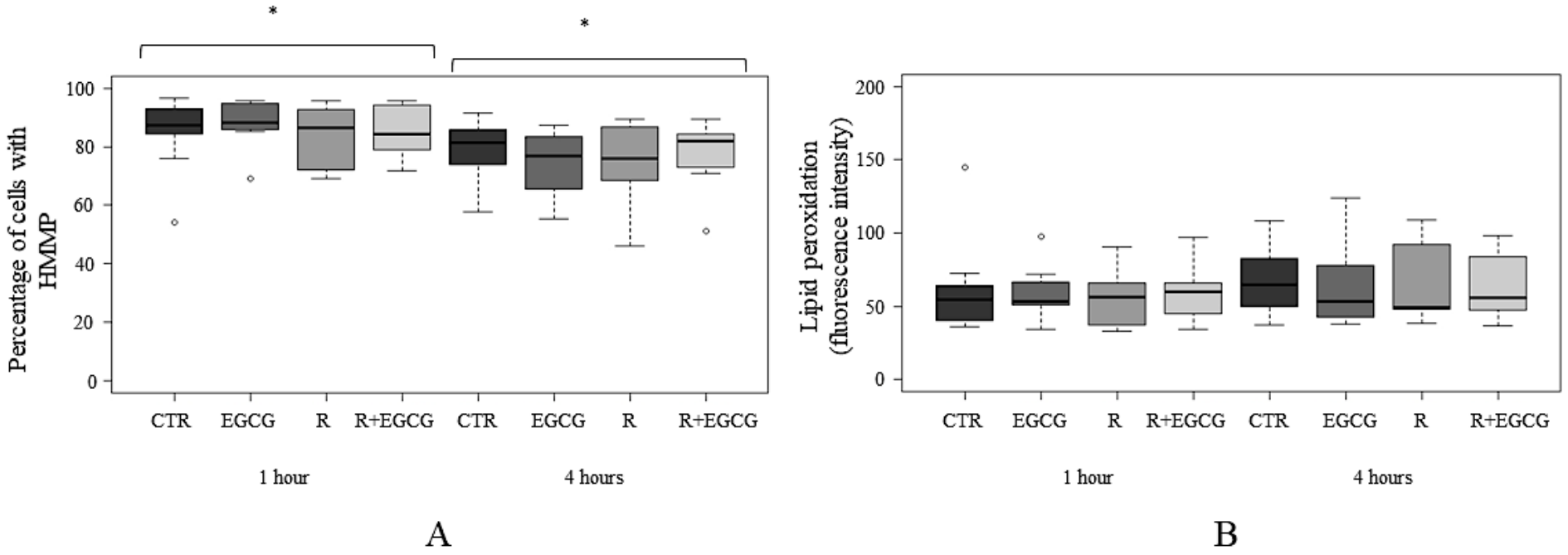
Figure 2. Sperm viability (panel A) and acrosome integrity (panel B) of frozen-thawed boar spermatozoa differently treated.



Horizontal line represents median; boxes represent 25-75 percentile; whiskers represent min-max, dots represent outliers. * indicates overall significant difference ($p<0.05$) between time 1h and time 4h. Four boars were used (one ejaculate each) with three replicates for each boar (n= 12). CTR: control; EGCG: Epigallocatechin-3-gallate 50 µM; R: Resveratrol 2mM.

Figure 3

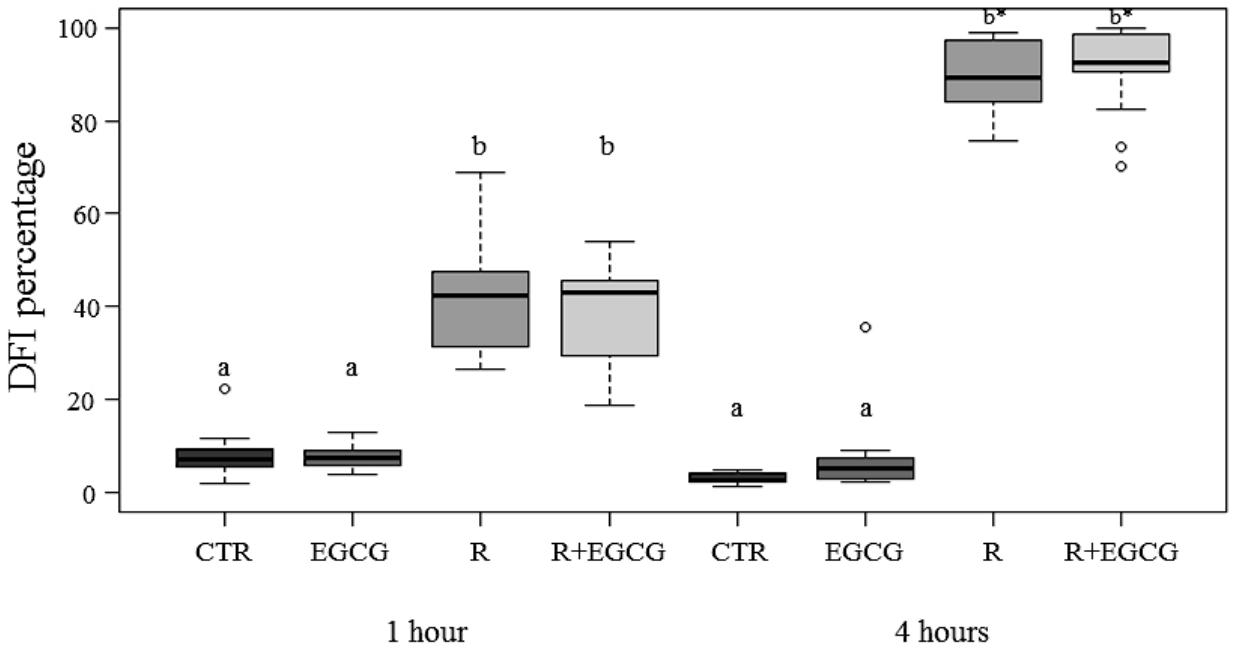
Figure 3. Percentage of living cells with high mitochondrial membrane potential (HMMP) (panel A) and lipid peroxidation of living cells membrane (mean fluorescence intensity of BODIPY 581/591) (panel B) of frozen-thawed boar spermatozoa differently treated.



Horizontal line represents median; boxes represent 25-75 percentile; whiskers represent min-max, dots represent outliers. * indicates overall significant difference ($p<0.05$) between time 1h and time 4h Four boars were used (one ejaculate each) with three replicates for each boar (n= 12). CTR: control; EGCG: Epigallocatechin-3-gallate 50 µM; R: Resveratrol 2mM.

675 **Figure 4**

676 Figure 4. DNA fragmentation index (DFI%) of frozen-thawed boar spermatozoa differently treated.

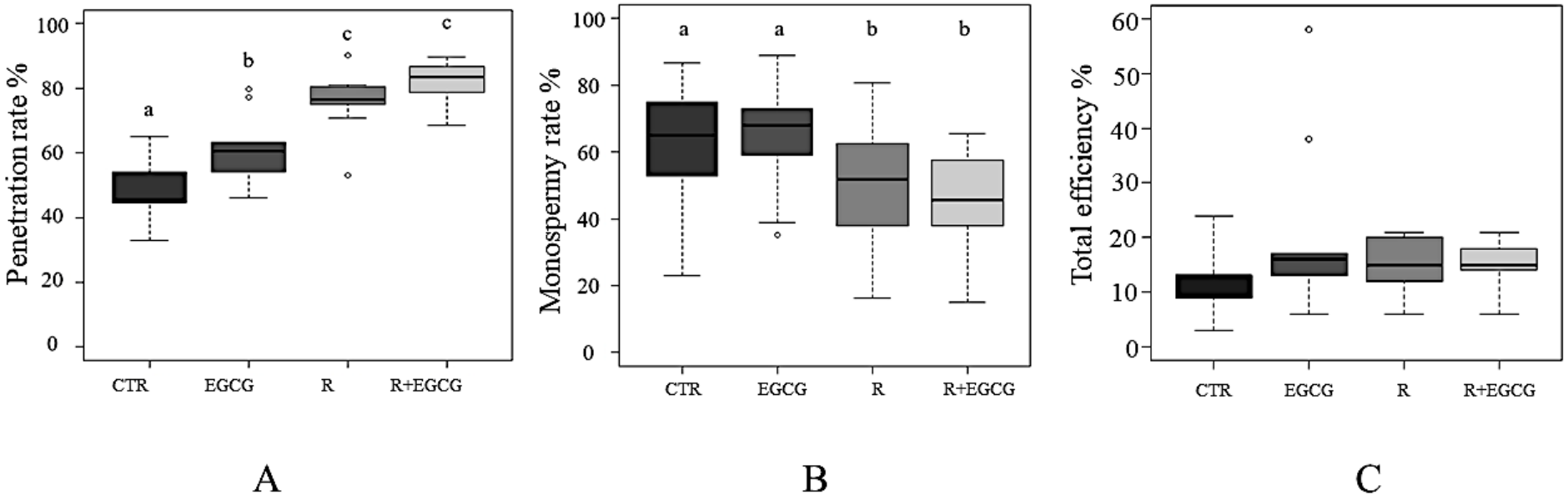


677

678 Horizontal line represents median; boxes represent 25-75 percentile; whiskers represent min-max,
679 dots represent outliers. Different superscripts indicate significant difference for $p<0.05$. * indicates
680 significant difference between 1h and 4 h ($p<0.05$). Four boars were used (one ejaculate each) with
681 three replicates for each boar (n= 12). Dots represent outliers. CTR: control; EGCG:
682 Epigallocatechin-3-gallate 50 µM; R: Resveratrol 2mM.

Figure 5

Figure 5. Penetration rate (panel A) and monospermy (panel B) and total efficiency (panel C) of porcine oocytes in vitro fertilized with differently treated semen samples.



Penetration rate: number of penetrated oocytes/total number of inseminated oocytes; monospermy rate: number of oocytes penetrated by one spermatozoon/total number of penetrated oocytes; total efficiency: number of oocytes penetrated by one spermatozoon/number of inseminated oocytes. Different superscripts indicate significant difference for $p<0.05$; horizontal line represent median; boxes represent 25-75 percentile; whiskers represent min-max. Three boars were used (one ejaculate each) with three replicates per boar (n=9). Dots represent outliers. CTR: control; EGCG: Epigallocatechin-3-gallate50 µM; R: Resveratrol 2mM.

692 **Table 1**

693 Table 1. Kinematic features of spermatozoa belonging to each cluster.

Parameter/Cluster	Slow non-progressive	Average	Rapid progressive	Rapid non-progressive
ALH	3.5±3.3	3.8±3.4	5.5±2.7	8.9±2.7
BCF	44.3±21.7	34.5±20.0	41.0±10.6	33.8±13.5
LIN	21.0±14.0	46.0±19.0	56.0±17.0	27.0±16.0
STR	55.0±32.0	87.0±14.0	91.0±10.0	58.0±30.0
VAP	21.4±18.4	44.2±25.4	94.4±28.9	82.7±26.9
VCL	55.5±48.6	81.9±56.7	151.8±56.7	176.7±54.3
VSL	10.7±12.3	37.4±21.6	81.5±23.0	47.4±26.4
Number of cells	5895	3891	4343	3824

694 Data represent median values ± interquartile range. ALH: amplitude of lateral head displacement
695 (μm); BCF: beat cross frequency (Hz); LIN: linearity %; STR: straightness %; VAP average path
696 velocity (μm/sec); VCL: curvilinear velocity (μm/sec); VSL: straight line velocity (μm/sec).

Table 2

Table 2. Cluster analysis results. Different distribution of spermatozoa subpopulation percentage depending on cluster and treatment.

Time	1 hour					4 hours			
Cluster	Slow non-progressive %	Average %	Rapid progressive %	Rapid non-progressive %		Slow non-progressive %	Average %	Rapid progressive %	Rapid non-progressive %
CTR	30.9	18.3	29.6	21.1		27.9	24.3	33.0	14.9
EGCG	31.2	18.3	25.8	24.8		31.0	23.3	25.5	20.2
R	44.4*	21.1	4.1*	30.3*		51.2*	29.8	1.2*	17.8
R+EGCG	41.5*	23.3	6.6*	28.5*		43.2*	32.1	3.9*	20.7

Each row represents the percentage of cells belonging to the different cluster (considering 100% the sum of percentage for each treatment). * indicate significant difference in column within each time (chi square test, $p<0.001$).

CTR: control; EGCG: Epigallocatechin-3-gallate; R: Resveratrol.

Table 3

Table 3. Percentages of tyrosine phosphorylated protein patterns (mean ± SD) in differently treated groups after 1 h of incubation in a capacitating medium.

	A	B	C	Neg
CTR	58.28±15.88	27.19±15.74	2.05±1.69	12.47±5.33
R	51.03±9.31	29.26±12.40	1.70±0.65	18.00±12.27
EGCG	53.34±15.32	31.64±13.43	2.78±1.89	12.23±5.23
R+EGCG	53.62±8.93	27.44±10.83	3.00±3.04	15.94±8.51

Different tyrosine phosphorylation patterns. A: spermatozoa with acrosome and equatorial sub segment positivity; B: spermatozoa with tail positivity (whole tail or the principal piece only) and acrosome and/or equatorial sub-segment positivity; C: spermatozoa with tail positivity (whole tail or principal piece only); and NEG: spermatozoa with no positive signal. Three boars were used (one ejaculate for each boar) and the analysis was repeated twice for each (n=6).

CTR: control; EGCG: Epigallocatechin-3-gallate; R: Resveratrol.

Supplementary file 1

Supplementary file 1. Sperm kinematics characteristics at 1h and 4 h incubation at 37°C with different treatments.

Time	1 hour				4 hours			
Variable	CTR	EGCG	R	R+EGCG	CTR	EGCG	R	R+EGCG
ALH	5.10±0.47^{a}	5.03±0.39^{a}	5.00±2.38^{a}	5.73±0.65^{b}	5.13±0.25^{a}	5.16±0.46^{a}	3.61±2.66^{c}	3.41±2.72^{c}
BCF	37.88±1.48^{a}	37.96±1.42^{a}	26.13±8.85^{b}	30.46±5.57^{b}	38.82±1.60^{a}	37.87±2.55^{a}	25.05±12.89^{b}	26.38±11.52^{b}
LIN	39.08±6.53^{a}	37.67±5.53^{a}	26.50±8.83^{b}	30.33±4.03^{b}	41.17±6.28^{a}	39.25±7.01^{a}	25.75±13.06^{b}	28.50±13.63^{b}
STR	70.67±6.21^{a}	68.58±4.74^{ab}	56.25±18.37^{b}	64.00±6.95^{b}	75.58±5.77^{a}	73.33±6.45^{a}	53.67±26.19^{b}	59.67±21.14^{b}
VCL	139.91±11.77^{a}	135.36±12.04^{a}	112.03±39.95^{b}	121.38±12.11^{b}	131.20±9.69^{a}	133.27±10.55^{a}	90.16±47.54^{c}	93.03±39.03^{c}
VAP	72.19±10.37^{a}	69.80±9.59^{a}	51.93±19.55^{b}	55.76±6.69^{b}	67.85±6.97^{a}	67.01±6.49^{a}	40.78±21.35^{c}	41.83±17.87^{c}
VSL	51.98±9.69^{a}	48.51±7.91^{a}	31.97±12.35^{b}	35.81±4.33^{b}	51.86±8.02^{a}	49.72±7.62^{a}	26.25±14.85^{b}	27.63±13.77^{b}

Sperm kinematics parameters: ALH amplitude of lateral head displacement (μM); BCF beat cross frequency (Hz); LIN linearity (%); STR straightness (%); VCL curvilinear velocity (μm/sec); VAP average path velocity (μm/sec); VSL straight line velocity (μm/sec).

Data represent mean ± SD. Different superscript within a row indicate significant difference for $p<0.05$.

CTR: control; EGCG: Epigallocatechin-3-gallate; R: Resveratrol.

717 **Supplementary file 2**

718 Supplementary file 2. Principal components loadings and variance.

	Comp.1	Comp.2	Comp.3	Comp.4	Comp.5	Comp.6	Comp.7
ALHs	-0.307	0.478	0.108	0.711	0.374	0.136	-0.036
BCFs	0.077	0.153	-0.980	0.059	0.059	-0.059	-0.008
LINs	-0.301	-0.537	-0.046	0.023	0.594	-0.438	0.273
STRs	-0.256	-0.544	-0.102	0.525	-0.513	0.000	-0.299
VAPs	-0.519	0.149	-0.021	-0.396	0.120	-0.130	-0.721
VCLs	-0.452	0.351	0.013	-0.066	-0.476	-0.495	0.443
VSLs	-0.520	-0.135	-0.123	-0.231	-0.010	0.725	0.344
Variances	3.301	2.193	0.966	0.306	0.146	0.069	0.020
SD	1.817	1.481	0.983	0.553	0.382	0.262	0.143
Proportion of variance	0.472	0.313	0.138	0.044	0.021	0.010	0.003
Cumulative variance	0.472	0.785	0.923	0.966	0.987	0.997	1.000

719 Principal components analysis' report of component loadings, variances and description of the
720 different impact of each single component (SD, proportion of variance and cumulative variance).

PAPER 3

"Epigallocatechin-3-gallate (EGCG) and green tea polyphenols do not improve stallion semen parameters during cooling at 4°C."

NOTICE:

This is the revised version of the manuscript published in Reproduction in Domestic Animals. The definitive version is:

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Revised

Epigallo-catechin-3-gallate (EGCG) and green tea polyphenols do not improve stallion semen parameters during cooling at 4°C.

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Abstract

Stallion semen storage for artificial insemination is mainly based on liquid cooled storage. In many stallions this technique maintains sperm quality for an extended period of time (24-72 h) at 7° C. While this technique is commonly used in the horse industry, there can be a decline in fertility in some stallions, due to an inability of their sperm to tolerate the cool-storage process. The aim of the present work was to evaluate the effect of two natural antioxidants (Epigallo-catechin-3gallate EGCG at 20, 60, 120 µM and green tea polyphenols, PLow, PMed, PHigh, at 0.001, 0.01 and 0.1 mg/mL) on some sperm parameters (sperm motility, viability/acrosome integrity, and DNA quality) in extended semen immediately after its collection (T0) and after 2, 6, 24 and 48 h of cool-storage. Two ejaculates from three trotter stallions were analyzed after 48 h of storage at 4° C. No beneficial effect on the analyzed parameters was observed: the two antioxidants were not able to improve sperm quality after 48 h of storage. These results are in agreement with previous findings on the effect of different antioxidants reported by other researches, who have demonstrated that stallion semen keep good antioxidant capacity after dilution for 24 h. In conclusion, the positive effect exerted by antioxidant molecules in other species is not confirmed in the equine one.

Introduction

Equine industry uses assisted reproduction techniques for breeding. One of these techniques, cool-shipped semen, reduces sperm metabolism through cooling and allows a short-term preservation of stallion semen so that it can be shipped while it is still maintaining a good quality (Aurich., 2008; Rigby et al., 2001).

Fertility in some stallions is dramatically reduced following the shipping process. The sperm quality of cool-shipped semen may be affected by semen collection technique, chemical composition of the extenders, centrifugation for removal/reduction of seminal plasma, cooling rate and storage (Rigby et al., 2001; Barbas et al., 2009; Cuervo-Arango et al., 2014; Aurich et al., 2007). Cooling semen exposes spermatozoa to stresses, generally known as "cold shock", resulting in a loss of cell viability, motility and, at the end, fertilizing ability (White, 1993). In addition, stallion sperm may be more susceptible than that from other species to cold shock due to the low cholesterol content of its plasma membrane (Darin-Bennett and White, 1977). These negative effects have been recognized to be caused also by hyper-production of reactive oxygen species (ROS), whose main target are membrane phospholipids and DNA (Ball et al., 2001a; García at al., 2011; Aurich et al., 1997). Spermatozoa are particularly sensitive to ROS, partially because, when deprived of seminal plasma, they lose most of their antioxidant apparatus; secondarily, stallion spermatozoa are highly dependent on oxidative phosphorylation for their metabolism (Bucci et al., 2011; Ball et al., 2001b) and, as a consequence, their mitochondria produce a high amount of ROS.

To date, several antioxidants have been tested to preserve stallion semen from oxidative stress with different and conflicting results. Resveratrol (Giaretta et al., 2013) did not give any result at low doses and exhibited negative effects at high doses, ascorbic acid (Aurich et al., 1997) showed a positive effect on sperm membrane integrity, while catalase did not exert any positive effect. Pyruvate exerted positive effects on sperm motility, while xanthurenic acid did not (Bruemmer et a., 2002); melatonin showed positive results on apoptosis like changes and lipid peroxidation (da Silva et al., 2011), and the addition of superoxide dismutase to the cooling extender was beneficial as well (Cocchia et al., 2011). Finally, quercetin exerted positive effects on stallion semen after freezing (Gibb et al., 2013). Green tea extracts contain the polyphenolic compound epigallocatechin-3-gallate (EGCG), a powerful antioxidant which removes free radicals by reacting with hydrogen, alkoxyl or peroxyl radicals and chelating iron (Plaza Davila et al., 2015). In addition, EGCG indirectly increases the antioxidant potential by removing free radicals and by stimulating catalase activity (Schroeder et al. 2008).

Recently, green tea phytocomplexes or polyphenols have been used in dog cooled semen (Wittayarat et al., 2012, 2013); an increase of spermatozoa lifespan and motility was observed.
The aim of the present study was to test the effect of different doses of EGCG and green tea extract polyphenols (P) on stallion sperm quality during storage at 4°C for 48 h.

Materials and methods

Unless otherwise stated, all chemicals were purchased from Sigma–Aldrich (Milan, Italy).

Experimental design

Semen collection and preparation

Two ejaculates were collected from three trotter stallions of proven fertility, individually housed at the National Institute of Artificial Insemination, University of Bologna, Italy, using a Missouri artificial vagina with an inner liner and- inline filter to separate the gel fraction (Nasco, Fort Atkinson, WI, USA). Ejaculates were immediately evaluated for volume (in a 50 mL Falcon tube) and concentration (NucleoCounter SP 100, Chemometec, Denmark). Semen was diluted to a final concentration of 30 x 10^6 spermatozoa/mL in Kenney's extender and divided into 6 aliquots: CTR (control); E20, E60 and E120 (EGCG at concentrations of 20, 60, 120 µM, respectively); PLow, PMed and PHigh (Polyphenols at a concentration of 0.001, 0.01 and 0.1 mg/mL respectively).
Sperm motility, viability with acrosome integrity and DNA integrity were assessed at 0, 2, 6 and 24 h of storage at +4°C.

Motility evaluation

Motility was measured using a computer-assisted sperm analysis system (CASA, Hamilton Thorne, IVOS Ver. 12, standard equine setting). Semen was extended to 30x10^6 sperm/mL, and 1000 cells were analyzed using a fixed-height Makler Chamber. Sperm motility endpoints assessed were: total motility percentage (TM), progressive motility percentage (PM), curvilinear velocity (VCL), straight-line velocity (VSL), average path velocity (VAP), straightness (STR), linearity (LIN) and beat cross frequency (BCF). The settings parameters of the program were the followings: frames per second 60, number of frames 45, minimum contrast 70, minimum cell size 4 pixel, cell size 6 pixel, cell intensity 106; threshold path velocity, 50 mm/s; threshold straightness, 70%; and path velocity cut off, 20 mm/s.

Viability and acrosome integrity

Sperm acrosome intactness assay (fluorescein-labeled Pisum sativum agglutinin [FITC-PSA]/propidium iodide [PI]) was used for evaluating viability and acrosome integrity; cytometry was performed using a FACS Calibur flow cytometer (Becton Dickinson, Milan, Italy) with a 488 nm argon-ion laser. Emission measurements were made using 530/30 band pass (green/FL-1) and 585/42 band pass (red/FL-2) filters. Debris was gated out using a forward scatter/side scatter dot plot, and a minimum of 5000 cells per sample was analyzed. Data were acquired using the CellQuest Pro software (Becton Dickinson). Viable acrosome-intact spermatozoa (VAI) were those cells that did not acquire PI and FITC-PSA, whereas nonviable sperm were those with fluorescence red because of PI uptake. Fifty microliters from each treatment sample were diluted with 133 µL of Dulbecco's PBS solution (Invitrogen Gibco, Carlsbad, CA, USA) and stained with 2 µL of PI (Invitrogen Molecular Probes, Eugene, OR, USA; 2.4 mM working solution) and 0.05 mg/mL Pisum sativum. Samples were incubated at room temperature in the dark for 10 min, and then 20 µL of the stained sample was mixed with 400 µL of Dulbecco's PBS solution and subjected to analysis. A flow rate of approximately 300 events/s was used, and a total of 5000 events per sample was evaluated. List-mode data were analyzed by WinList software (Verity Software House, Topsham, ME, USA).

Sperm chromatin structure assay

Sample preparation and processing, as well as flow cytometer adjustments, were performed as previously described (Evernon et al., 2002; Love, 2005). Briefly, an aliquot of each semen sample was immediately frozen and stored at -20°C until analysis (maximum 2 weeks). The sperm samples were handled individually and were thawed in a 37 °C water bath. Immediately after thawing (30–60 sec), 2 to 7 µL aliquots of semen were diluted to 200 µL in a buffer solution (0.186-g disodium EDTA, 0.790-g Tris-HCI, 4.380-g NaCI in 500-mL deionized water, pH 7.4). This was mixed with 400 µL of acid detergent solution (2.19-g NaCl, 1.0 mL of 2N HCI solution, 0.25-mL Triton X, deionized water quantum sufficit to a final volume of 250 mL). After 30 sec, 1.2 mL of the acridine orange solution were added (3.8869 g citric acid monohydrate, 8.9428 g Na2HPO4, 4.3850 g NaOH, 0.1700 g disodium EDTA, 4 mg/mL acridine orange stock solution (1 mg/mL), water quantum sufficit to 500-mL, pH 6.0). The sample was covered with aluminum foil, placed in the flow cytometer and allowed to pass through the tubing for 30 sec before counting of the cells. The cell flow rate was placed on the low setting for the machine which, based on sperm concentration in the solution, resulted in an actual flow rate of 100 to 200 cells/s. A total of 5000 events was evaluated for each sample. Sperm from a single control stallion were used as a biologic control to standardize instrument

settings between days of use. The flow cytometer was adjusted such that the mean green fluorescence was set at 500 channels (Fl-l at 500) and mean red fluorescence at 150 channels (Fl-3 at 150). Data were acquired in a list mode, and analysis was performed using WinList software (Verity Software House). The percent of sperm with abnormal DNA was defined by the parameter DNA fragmentation index (DFI).

Statistical analysis

Data were analyzed by R version 3.2.2 (R Foundation for Statistical Computing, Vienna, Austria). After checking for normal distribution, data were analyzed by an ANOVA test followed by the Tukey post hoc test for multiple comparison. Data are expressed as mean ± standard deviation.
In all statistical analyses, the minimal level of significance was set at $p<0.05$ unless otherwise specified.

Results

Some parameters showed a decrease due to storage time: TM, PM, BCF for motility (see Tables 1, 2, 7) and DNA damage (Table 11). The other parameters, regarding motion characteristics (VCL, VAP, VSL, ALH, STR, LIN) and the percentage of live spermatozoa with intact acrosomes were not negatively influenced by storage time. See Tables 3, 4, 5, 6, 8, 9, 10.

Discussion

It is generally assumed that equine spermatozoa survive quite well at 4°C during the first 24 h of storage; then, for some stallions, viability dramatically decreases. Together with the viability drop, also other parameters (such as sperm motility, DNA fragmentation, mitochondrial activity) undergo a loss, as described by Ball et al. (2001a).
Many Authors reported that oxidative stress is one of the main problems stallion spermatozoa undergo during storage (Ball et al., 2001b; Garcìa et al., 2011; Aurich et al., 1997) and that the relative low cholesterol content in stallion spermatozoa membrane (Darin-Bennett and White, 1977) could exert negative effects on the storage outcome.
Different antioxidants have been tested on chilled stallion spermatozoa to minimize the adverse effects of oxidative stress and to improve sperm quality, but the results obtained are sometimes limited or unsatisfactory. In the present study, we tested different doses of EGCG and green tea extract polyphenols (plant molecules recognized to exert beneficial effects on spermatozoa; Plaza Davila et al., 2015, Wittayarat et al., 2012, 2013) on stallion sperm stored at 4°C for 48 h.

Our results demonstrated a consistent decrease in some spermatozoa parameters: total and progressive motility, beat cross frequency and DNA fragmentation undergo significant changes during the storage period, usually more evident at 24 and 48 h. On the other hand, our results clearly show that EGCG and the green tea extracts are not effective in reducing the cooling/storage damage: in fact, we did not register any improvement of any of the parameters we analyzed and the decline in sperm quality did not seem to be influenced by the addition of these substances.

One of the most plausible explanation for these results is that we did not eliminate seminal plasma from sperm suspension: we only added a defined medium with or without antioxidants to reach the fixed concentration. It is likely that seminal plasma contents (catalase, sodium-peroxide dismutase, vitamin C, selenium, zinc) continues playing a protective role during sperm storage and that the addition of exogenous molecules does not further improve sperm quality after storage (Ball et al., 2001b; Garcìa et al., 2011 Aurich et al., 1997; Bucci et al., 2011; Ball et al., 2001 a; Giaretta et al., 2013).

In a preliminary experiment, higher concentrations of the two antioxidants have been tested; in particular, green tea extract was used at 0.5, 0.75 and 1.0 mg/mL, as reported in dog by Wittayarat et al. (2012, 2013). These concentrations demonstrated to be toxic for stallion sperm, as 6 h after resuspension a significant decline in sperm motility and viability was observed, with a 100% dead immotile spermatozoa after 24 h (data not shown). We therefore reduced the polyphenol supplementation the toxic concentration. Other authors (Wittayarat et al. 2012, 2013) used the polyphenol extract for a very long storage period (4 weeks at 5°C); these Authors did not find any difference in viability and acrosome integrity in the first study (Wittayarat et al. 2012) while observing a positive effect on viability in the second one (Wittayarat et al., 2013). The results from these two studies are in agreement with those from the present one, as we did not notice any difference in viability and acrosome integrity parameters during 48 h storage at 4°C. As for motility, we did not notice any effect of either P or EGCG and we registered a decrease in total and progressive motility. At this regard it should be pointed out that Wittayarat et al. (2012, 2013) assessed motility in a subjective way and obtained an indirect motility index, while in our study we assessed motility by a CASA system, that is essentially objective. In addition, those Authors examined their samples during a very long period of time, up to four weeks, and registered some differences at the endpoint of their experimental period, while in the earlier time-points they did not register any difference either in viability or in motility.

It has to be stressed that some species-specific features of spermatozoa do not guarantee the same effects of antioxidants across species.

We already demonstrated that Resveratrol is ineffective for improving stallion sperm storage (Giaretta et al., 2013), and that it could even become dangerous if supplemented at high concentrations. Other authors obtained similar results in porcine sperm (Martin-Hidalgo et al., 2013), concluding that this antioxidant is not suitable for sperm preservation.

On the basis of the above cited references (Giaretta et al., 2013; Ball et al., 2001b) and of our results, we may infer that, at least for cooled equine semen, supplementation of the storage medium with natural antioxidants is not useful, in that they seem to exert a negative effect when used at too high concentrations. Other Authors (Kankofer et al., 2005) indeed, demonstrated that diluting seminal plasma (in commercial extenders) for stallion sperm storage at 5°C activates the antioxidant activity of some enzymes, naturally present in seminal plasma from this species, and reduces ROS generation at 24 h. Another study (Pagl et al., 2006) showed that the addition of glutathione peroxidase, superoxide dismutase (SOD) and catalase to extended stallion semen does not improve sperm quality after storage. Those Authors concluded that ROS couldn't be a cause of decreased sperm quality in liquid stored stallion spermatozoa. In contrast, the addition of antioxidant molecules, such as SOD and melatonin, were effective in improving the quality of cool-stored stallion sperm (da Silva et al., 2011; Cocchia et al., 2011).

In a recent study (Plaza Davila et al., 2015) we demonstrated that EGCG helps in protecting stallion sperm function from the negative effect induced by rotenone during in vitro induced capacitation. In that study we did not find any difference in sperm quality parameters (acrosome reaction and viability) between treatments, but we did observe an important difference in heterologous binding capacity which was decreased by rotenone but fully restored by EGCG. These results could indicate that sperm viability, acrosome integrity, motility and DNA integrity are not affected by the antioxidants both in preservation and capacitating media.

Therefore, it is reasonable to hypothesize that oxidative stress in cooled equine semen is low, as reported by Pagl and co-workers (2006), while it could represent an important limiting factor in cryopreservation (Ortega-Ferrusola et al., 2009).

The effect of antioxidants may vary depending on species as well as on the way semen is processed for preservation (cooled vs. frozen-thawed). Therefore, it is possible that an antioxidant could be effective in chilled semen but not in frozen one, or effective in dog sperm but not in stallion sperm.

In conclusion, no positive effect of EGCG or polyphenol extract was registered in cooled stallion semen up to 48 h storage, and therefore the addition of these substances in cooling medium for stallion sperm storage is not useful.

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Table 1

Table 1. Total motility percent comparing treatments within time points for 6 ejaculates (2 ejaculates per stallion; mean ± standard deviation). Abbreviations: CTR – control; E20, E60, E120; 20, 60, 120 µM EGCG; Plow, PMed, PHigh; Polyphenol 0.001, 0.01 and 0.1 mg/ml. Different superscript in column indicate significant difference within time ($p<0.05$).

	Treatment						
Time	CTR	E20	E60	E120	PLow	PMed	PHigh
0h	81,71±7.78 [a]	84,86±4.78 [a]	81,57±12.74 [a]	80,67±6.19 [a]	81,60±8.17 [a]	81,50±10.75 [a]	82,14±9.3 [a]
2h	82,71±4.89 [a]	81,14±8.73 [a]	82,29±5.31 [a]	83,33±8.33 [a]	80,83±10.72 [a]	83,83±7.33 [a]	80,86±4.18 [a]
6h	78,43±6.80 [a]	79,71±8.06 [a]	82,71±5.82 [a]	80,17±6.08 [a]	77,83±7.63 [a]	77,83±5.85 [a]	78,43±10.18 [a]
24h	69,14±12.01 [bc]	66,57±8.12 [bc]	67,00±11.50 [bc]	64,67±16.15 [bc]	69,33±12.08 [bc]	67,50±7.84 [bc]	67,29±8.20 [bc]
48h	49,86±13.40 [c]	51,14±15.16 [c]	49,14±11.81 [c]	51,00±9.65 [c]	55,17±13.93 [c]	54,50±14.11 [c]	51,43±9.52 [c]

296 **Table 2**

297 Table 2. Progressive motility percent comparing treatments within time points for 6 ejaculates (2 ejaculates per stallion; mean ± standard deviation).
298 Abbreviations: CTR – control; E20, E60, E120; 20, 60. 120 µM EGCG; Plow, PMed, PHigh; Polyphenol 0.001, 0.01 and 0.1 mg/ml. Different superscript
299 in column indicate significant difference within time ($p<0.05$).

	Treatment						
Time	CTR	E20	E60	E120	PLow	PMed	PHigh
0h	42,14±9.28 [a]	43,14±11.64 [a]	41,86±11.51[a]	41,50±13.41[a]	41,20±10.38 [a]	41,00±11.59 [a]	40,43±11.15 [a]
2h	35,00±11.49 [b]	34,57±9.27 [b]	36,14±13.92 [b]	35,00±8.99 [b]	35,83±9.28 [b]	40,00±12.46 [b]	32,43±6.97 [b]
6h	26,71±6.55 [c]	28,43±3.46 [c]	29,00±5.45 [c]	27,33±3.08 [c]	29,00±6.48 [c]	28,50±5.96 [c]	26,14±5.11 [c]
24h	20,57±6.19 [d]	20,57±5.32 [d]	20,43±4.86 [d]	19,00±6.36 [d]	20,33±8.76 [d]	20,00±7.40 [d]	20,14±5.76 [d]
48h	14,57±4.93 [d]	15,43±6.35 [d]	14,00±6.61[d]	13,17±7.25 [d]	19,00±10.12 [d]	17,50±9.77 [d]	14,86±7.56 [d]

300

Table 3

Table 3. Curvilinear velocity (VCL) (µm/sec) comparing treatments within time points for 6 ejaculates (2 ejaculates per stallion; mean ± standard deviation). Abbreviations: CTR – control; E20, E60, E120; 20, 60. 120 µM EGCG; Plow, PMed, PHigh; Polyphenol 0.001, 0.01 and 0.1 mg/ml.

	Treatment						
Time	CTR	E20	E60	E120	PLow	PMed	PHigh
0h	199,96±18,03	200,99±26,68	198,69±25,38	200,65±33,81	205,94±32,12	200,68±27,10	201,11±25,66
2h	221,91±25,73	208,79±17,11	216,66±2,38	217,52±14,66	195,75±46,16	210,07±27,09	214,11±12,55
6h	227,29±23,68	218,13±22,99	218,66±13,83	224,37±16,45	217,87±22,78	214,08±25,71	222,63±19,55
24h	205,29±34,48	207,30±31,90	213,39±28,93	224,13±38,88	213,83±39,87	216,25±42,08	219,07±37,89
48h	195,34±53,53	208,06±26,08	202,91±25,14	211,78±35,71	206,73±31,86	205,10±28,71	203,13±25,24

Table 4

Table 4. Average path velocity (VAP) (µm/sec) comparing treatments within time points for 6 ejaculates (2 ejaculates per stallion; mean ± standard deviation). Abbreviations: CTR – control; E20, E60, E120; 20, 60. 120 µM EGCG; Plow, PMed, PHigh; Polyphenol 0.001, 0.01 and 0.1 mg/ml.

	Treatment						
Time	CTR	E20	E60	E120	PLow	PMed	PHigh
0h	112,71±15,57	112,61±17,76	114,00±19,12	112,72±22,69	115,44±25,06	112,05±16,94	111,49±19,28
2h	118,49±12,18	113,40±8,83	117,80±11,55	117,42±10,45	109,77±19,26	114,77±17,02	116,49±8,19
6h	119,01±19,48	114,23±14,54	115,17±12,61	117,17±16,16	115,78±16,21	112,12±15,81	116,77±12,75
24h	99,70±20,52	103,44±15,02	105,57±14,52	111,28±20,39	106,40±19,46	106,72±20,26	109,63±18,25
48h	93,60±25,61	102,20±12,91	97,64±9,55	101,10±18,35	102,43±14,59	98,68±11,04	96,64±11,39

309 **Table 5**

310 Table 5. Straight-line velocity (VSL) (µm/sec) comparing treatments within time points for 6 ejaculates (2 ejaculates per stallion; mean ± standard
311 deviation). Abbreviations: CTR – control; E20, E60, E120; 20, 60. 120 µM EGCG; Plow, PMed, PHigh; Polyphenol 0.001, 0.01 and 0.1 mg/ml.

	Treatment						
Time	CTR	E20	E60	E120	PLow	PMed	PHigh
0h	79,07±17,46	77,93±16,36	79,77±17,43	77,32±20,92	81,00±22,32	76,60±16,08	76,59±18,83
2h	75,03±12,16	73,56±13,42	74,31±16,07	75,42±16,07	71,97±13,17	74,92±18,61	71,66±12,12
6h	67,76±16,85	67,53±10,87	68,10±13,76	67,80±12,30	69,28±12,90	67,85±11,29	66,49±11,79
24h	55,37±8,95	57,99±4,58	57,76±6,60	58,73±4,10	58,15±6,50	56,85±5,13	58,56±5,33
48h	50,83±8,20	55,96±6,02	51,26±4,45	50,77±5,95	58,10±9,18	53,92±6,88	51,90±6,28

312

313 **Table 6**

314 Table 6. Amplitude of lateral head displacement (ALH) (µm)comparing treatments within time points for 6 ejaculates (2 ejaculates per stallion; mean ±
315 standard deviation). Abbreviations: CTR – control; E20, E60, E120; 20, 60. 120 µM EGCG; Plow, PMed, PHigh; Polyphenol 0.001, 0.01 and 0.1 mg/ml.

	Treatment						
Time	CTR	E20	E60	E120	PLow	PMed	PHigh
0h	7,24±0,82	7,14±0,42	7,06±0,48	6,95±0,66	7,50±0,53	7,15±0,47	7,23±0,48
2h	7,71±1,32	7,39±0,31	7,20±0,45	7,60±0,62	10,43±6,96	7,17±0,28	7,29±0,29
6h	7,76±0,59	7,51±0,48	7,70±0,54	7,88±0,43	7,43±0,39	7,78±0,53	7,54±0,46
24h	8,24±0,65	8,00±0,91	7,97±0,80	8,17±1,28	8,25±1,19	7,88±0,81	8,00±0,89
48h	8,21±1,97	7,84±0,88	7,99±1,04	8,60±1,01	8,18±0,66	8,02±0,65	8,30±0,66

316

317 **Table 7**

318 Table 7. Beat cross frequency (BCF) (Hz) comparing treatments within time points for 6 ejaculates (2 ejaculates per stallion; mean ± standard deviation).
319 Abbreviations: CTR – control; E20, E60, E120; 20, 60. 120 µM EGCG; Plow, PMed, PHigh; Polyphenol 0.001, 0.01 and 0.1 mg/ml. Different superscript
320 in columns indicate significant difference within time ($p<0.05$).

	Treatment						
Time	CTR	E20	E60	E120	PLow	PMed	PHigh
0h	37,56±2,28 ^{ab}	37,14±3,09 ^{ab}	38,33±2,63 ^{ab}	38,25±2,22 ^{ab}	37,10±4,68 ^{ab}	37,98±3,44 ^{ab}	36,40±3,41 ^{ab}
2h	38,29±2,86 ^{a}	38,83±1,54 ^{a}	39,47±1,75 ^{a}	39,07±2,47 ^{a}	32,50±14,57 ^{a}	38,95±3,09 ^{a}	39,23±2,13 ^{a}
6h	38,34±3,85 ^{a}	38,79±3,10 ^{a}	39,00±2,14 ^{a}	38,82±3,83 ^{a}	38,68±3,45 ^{a}	38,55±3,45 ^{a}	39,01±2,25 ^{a}
24h	35,24±3,57 ^{bc}	35,69±2,29 ^{bc}	35,71±2,53 ^{bc}	35,75±3,06 ^{bc}	35,55±2,83 ^{bc}	36,72±2,88 ^{bc}	36,91±2,45 ^{bc}
48h	33,26±5,46^{c}	34,71±3,65 ^{c}	33,64±3,67 ^{c}	33,42±3,76 ^{c}	36,12±4,38 ^{c}	33,50±2,47 ^{c}	33,30±2,24 ^{c}

321

Table 8

Table 8. Straightness (STR) percent comparing treatments within time points for 6 ejaculates (2 ejaculates per stallion; mean ± standard deviation). Abbreviations: CTR – control; E20, E60, E120; 20, 60. 120 µM EGCG; Plow, PMed, PHigh; Polyphenol 0.001, 0.01 and 0.1 mg/ml.

	Treatment						
Time	CTR	E20	E60	E120	PLow	PMed	PHigh
0h	69,71±5,79	69,00±7,59	69,43±7,07	69,00±7,72	69,20±6,30	68,33±7,26	68,29±7,23
2h	64,00±8,64	64,86±8,25	63,86±9,74	63,83±8,06	66,00±8,10	65,83±10,17	61,71±6,42
6h	58,29±5,50	59,86±5,64	59,71±5,31	58,67±3,14	60,67±6,12	60,67±6,25	58,14±6,15
24h	56,86±3,93	58,00±5,86	56,57±3,95	55,50±7,09	57,17±8,80	56,00±8,20	56,14±7,17
48h	57,43±11,57	57,14±8,73	55,00±7,70	53,00±10,37	58,00±9,76	57,00±10,64	56,14±9,23

Table 9

Table 9. Linearity (LIN) percent comparing treatments within time points for 6 ejaculates (2 ejaculates per stallion; mean ± standard deviation). Abbreviations: CTR – control; E20, E60, E120; 20, 60. 120 µM EGCG ; Plow, PMed, PHigh; Polyphenol 0.001, 0.01 and 0.1 mg/ml.

	Treatment						
Time	CTR	E20	E60	E120	PLow	PMed	PHigh
0h	42.00±5.13	41.14±5.76	42.29±5.71	41.67±6.25	41.00±5.70	40.50±5.68	40.29±5.96
2h	37.29±6.16	37.29±5.94	37.14±6.91	37.00±6.81	40.67±11.29	38.17±7.05	36.29±4.61
6h	32.29±4.64	33.57±3.41	33.57±5.13	32.50±3.28	34.00±4.47	33.67±3.83	32.57±3.95
24h	28.71±2.36	30.57±3.64	29.29±2.98	28.83±4.58	29.67±4.89	28.67±4.37	29.29±4.61
48h	29.57±8.58	29.43±5.22	27.86±5.15	26.33±6.19	30.17±6.62	28.33±6.59	27.71±5.41

Table 10

Table 10. Viability and acrosome intactness percent comparing treatments within time points for 6 ejaculates (2 ejaculates per stallion; mean ± standard deviation). Abbreviations: CTR – control; E20, E60, E120; 20, 60. 120 µM EGCG; Plow, PMed, PHigh; Polyphenol 0.001, 0.01 and 0.1 mg/ml.

	Treatment						
Time	CTR	E20	E60	E120	PLow	PMed	PHigh
0h	56.35±10.11	60.67±9.81	58.08±9.75	57.32±9.32	58.12±9.73	58.71±10.75	58.74±7.71
2h	54.18±6.82	56.06±8.41	56.22±7.09	53.63±8.78	55.17±7.77	55.04±9.27	55.13±6.64
6h	54.54±6.19	56.04±7.28	56.27±5.77	55.94±5.53	56.65±5.24	56.45±5.63	56.39±5.98
24h	54.53±7.13	59.02±7.39	58.85±7.51	58.01±7.69	60.25±7.51	60.11±7.14	58.02±7.43
48h	49.72±14.11	56.41±8.26	56.51±6.13	53.91±9.64	59.89±5.53	58.19±5.17	57.65±5.28

Table 11

Table 11. Chromatin damage percentage comparing treatments within time points for 6 ejaculates (2 ejaculates per stallion; mean ± standard deviation). Abbreviations: CTR – control; E20, E60, E120; 20, 60. 120 µM EGCG; Plow, PMed, PHigh; Polyphenol 0.001, 0.01 and 0.1 mg/ml. Different superscript in column indicate significant difference within time ($p<0.05$).

	Treatment						
Time	CTR	E20	E60	E120	PLow	PMed	PHigh
0h	24,63±7.24 [a]	30,61±10.01 [a]	30,84±11.37 [a]	31,03±11.52	31,46±11.90	29,74±8.60	27,42±7.74
2h	29,53±10.67 [ab]	40,26±25.74 [ab]	39,67±27.26 [ab]	40,73±28.15 [ab]	43,22±24.84 [ab]	40,62±29.90 [ab]	37,76±22.38 [ab]
6h	38,87±23.52 [b]	43,15±27.89 [b]	41,49±22.98 [b]	44,27±28.03 [b]	44,26±27.88 [b]	38,38±24.91 [b]	38,22±22.35 [b]
24h	42,30±25.90 [b]	38,31±20.0 [b]	39,47±14.95 [b]	43,54±24.13 [b]	44,23±21.16 [b]	38,00±14.90 [b]	34,85±13.34 [b]
48h	42,05±20.51 [b]	42,91±16.95 [b]	40,15±14.10 [b]	41,98±15.05 [b]	47,41±15.46 [b]	45,95±22.25 [b]	45,59±19.29 [b]

PAPER 4

"Photo-stimulation through LED prior to cryopreservation improves the cryotolerance of poor freezability boar ejaculates"

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Title

Photo-stimulation through LED prior to cryopreservation improves the cryotolerance of poor freezability boar ejaculates

Running title

Photo-stimulation and boar sperm cryopreservation

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Abstract

The present study was aimed to evaluate whether photo-stimulation of boar sperm before cryopreservation could increase their cryotolerance and, thus, positively affect their function and survival. A main inconvenient of boar sperm cryopreservation, as it occurs in other species, is the individual variability of sperm cryotolerance which underlies the existence of good (GFE) and poor freezability ejaculates (PFE). Therefore, the current study also evaluated whether the potential impact of photo-stimulation differs between GFE and PFE. Boar seminal doses were photo-stimulated before cryopreservation through MaxiPig® equipment which is a controlled-temperature device (set at 17ºC) with triple LED that emits at a wavelength range of 620-630 nm and irradiates the samples for 30 min following a pattern of 10 min of light, 10 min of darkness and 10 min of further light. Sperm motility, viability, acrosome integrity and mitochondrial membrane potential were evaluated 30 and 180 min post-thaw. LED photo-stimulation procedures increased the resilience of PFE to withstand cryopreservation, especially when viability and acrosome integrity and mitochondrial activity were evaluated at 180 minutes post-thaw. On the contrary, photo-stimulation had no effect on GFE and a detrimental effect on total sperm motility was observed in both types of ejaculates.

In conclusion, these results indicate that photo-stimulation may be considered as a potential tool to increase the cryotolerance of PFE but further research on the mechanisms underlying the detrimental effects on sperm motility is required, as this may allow to understand whether such increase in cryotolerance has a significant impact upon the fertilizing ability of PFE.

Keywords: Sperm; Boar; Cryopreservation; Photo-stimulation

1. Introduction

Sperm cryopreservation is the most efficient method for long term-storage of sperm in mammalian species (Holt, 2000). However, porcine artificial insemination (AI) performed with cryopreserved semen is still sub-optimal in terms of fertility and economic costs when compared with liquid-stored semen. Worldwide, less than 1% of all AI are conducted using frozen-thawed doses (Knox, 2016; Rodríguez-Gil and Estrada, 2013). While cryopreservation causes several damages on sperm function and integrity, it is the only method that allows for the creation of banks of genetic material of high hereditarily breeding males (Yeste et al., 2017). For this reason, several authors have tried to increase sperm survival at post-thaw through different approaches, such as supplementation of freezing and thawing media (see Yeste, 2016; Yeste et al., 2017 for reviews). In the context, photo-stimulation,

which has previously been shown to increase different sperm parameters in liquid-stored semen (Yeste et al., 2016), could also improve boar sperm cryotolerance.

Photo-stimulation consists of irradiating cells with light to modify their metabolism. During the last two decades of the 20th century, the biological effects of photo-stimulation were studied in many cellular tissues and also on gametes (Abdel-Salam and Harith, 2015). The first work on spermatozoa irradiation occurred in 1969 when Goldstein et al. light-stimulated starfish and sea-urchin spermatozoa using pulsed ruby laser micro-beam (Goldstein, 1969). Further studies focused on the light-effects on mammalian sperm cells using different types of low-power lasers. In summary, photo-stimulation has been found to: (1) increase cell energy supply via ATP amount; (2) improve motility of fresh semen; and (3) control the level of bacterial contamination in seminal doses (reviewed in Abdel-Salam and Harith, 2015).

With regard to frozen-thawed sperm, previous studies conducted in different species have provided interesting results. For example, irradiating cryopreserved avian sperm with a He–Ne laser maintained better their motility at post-thaw (Iaffaldano et al., 2013). In rams, photo-stimulation of frozen-thawed sperm better maintained their motility, viability and mitochondrial function (Nicolae et al., 2015), and a clear interaction between mitochondria and laser-light was observed (Iaffaldano et al., 2016). In bulls, low-laser irradiation of frozen-thawed sperm also preserved better their viability and acrosome integrity, even when incubated under capacitating conditions (Ocaña-Quero et al., 1997; Fernandes et al., 2015). Despite all of these results, the effects of photo-stimulation in pigs have been studied in liquid-stored but not in frozen-thawed sperm. Notwithstanding, exposing liquid-stored boar semen to light emission diode (LED)-based red light (wavelength range: 620nm-630nm) improves, perhaps via a mechanism related to mitochondrial function, sperm motility and mitochondrial membrane potential, and increases the fertilizing ability of boar seminal doses (Yeste et al., 2016). In this context, it is worth noting that the aforementioned study did not use a laser as light-source but rather LED, as this system is cheaper and easier to use than conventional lasers. In this way, the exposure of semen to light could be performed not only on laboratory conditions but also in the daily routine of commercial AI centers.

Against this background, the present study sought to address whether photo-stimulation of boar sperm before cryopreservation could increase their cryotolerance and, thus, positively affect its function and survival. A main inconvenient of boar sperm cryopreservation, as it occurs in other species, is the individual variability of sperm cryotolerance which underlies the existence of good (GFE) and poor freezability ejaculates (PFE) (Estrada, Rivera del Álamo, et al., 2017; Estrada, Rodríguez-Gil, et al.,

2017; Yeste et al., 2013, 2014). Therefore, the current study also evaluated whether the potential impact of photo-stimulation differed between GFE and PFE.

2. Materials and Methods

2.1. Semen samples

A total of 16 semen samples, each coming from a different boar, were used in the current study. All semen samples were obtained from a local farm (Servicios Genéticos Porcinos S.L.; Roda de Ter, Barcelona, Spain). According to the farm, boars were collected through the gloved-hand technique twice a week and were given a three-day period between collections. Sperm-rich fractions were either diluted with Androstar extender (Minitub Ibérica S.L., Tarragona, Spain) to a final amount of 3×10^9 per dose in doses of 80 mL. After dilution samples were cooled down to 17ºC and then transported in our laboratory within 4 h of collection.

Upon arrival at our laboratory, all samples were confirmed to be over the sperm quality standards (total motile sperm ≥80%, viable sperm ≥80%, morphologically normal spermatozoa ≥ 85%). Samples were photo-stimulated and cryopreserved the following day.

2.2. Photo-stimulation procedures

Seminal doses, prepared as described above, were photo-stimulated through MaxiPig® equipment (GenIUL, S.L.; Terrassa, Spain), which is a controlled-temperature device (set at 17ºC) with triple LED that emits at a wavelength range of 620 nm-630 nm and irradiates the samples for 30 min following a pattern of 10 min of light, 10 min of darkness and 10 min of further light. This machine was designed by this company after a previous study in fresh boar semen (Yeste et al., 2016). Control samples were kept at the same conditions without irradiation and sperm were cryopreserved and thawed following the routine protocol, as described in Section 2.3.

2.3. Semen cryopreservation and thawing

Sperm cryopreservation was conducted following Casas et al., 2009 protocol. Briefly, control and photo-stimulated semen doses were split into falcon tubes and centrifuged at 640×g at 17ºC for 5 min. The supernatant was removed and cell pellets were reunited and mixed with LEY (Lactose-Egg Yolk) medium at 17ºC to reach a final concentration of 1.5×10^9 spermatozoa·mL^{-1}. At this point, semen samples were cooled down to 5ºC and added with LEYGO (LEY+6% glycerol+1.5% Orvus Es Paste) medium to a final concentration of 1.0×10^9 spermatozoa mL^{-1}. Semen was packaged in 0.5-mL straws

and frozen with a controlled-rate programmable freezer (Icecube 14S; Minitub Ibérica S.L.) applying the following program: at -6°C·min^{-1} from 5°C to -5°C; at -39.82°C·min^{-1} from -5°C to -80°C; at -80°C (no decrease) for 30 sec; and -60°C·min^{-1} from -80°C to -150°C. Boar sperm straws were stored in liquid Nitrogen (N_2) at -196ºC for one week before evaluation.

Straws were thawed by shaking in a water bath at 38°C for 20 seconds and subsequently diluted with three volumes of Beltsville Thawing Solution (BTS) at the same temperature. Semen samples were maintained in the water bath at 38°C with the cap of the tube opened to allow air exchange. Sperm motility, viability, acrosome integrity and mitochondrial membrane potential were evaluated after 30 and 180 min of thawing.

2.4. Sperm motility assessment

Sperm motility was assessed using a computer-assisted sperm-analysis (CASA) system (Integrated Sperm Analysis System V1.0; Proiser S.L.; Valencia, Spain). With this purpose, a 5-µL drop was placed onto a Makler chamber (Sefi Medical Instruments; Haifa, Israel), previously warmed at 38ºC, and samples were evaluated under a phase-contrast microscope at 100× (Olympus BX41 microscope, Olympus Europe GmbH, Hamburg, Germany). Separate fields were captured and, at least, 1000 spermatozoa were evaluated. Two replicates were performed for each treatment. The following sperm parameters were recorded: total motility (TM, %), progressive motility (PM, %), curvilinear velocity (VCL, µm·s^{-1}), straight-line velocity (VSL, µm·s^{-1}), average path velocity (VAP, µm·s^{-1}), linearity (LIN, %), straightness (STR, %), oscillation (WOB, %), amplitude of lateral head displacement (ALH, µm) and beat cross frequency (BCF, Hz). A boar spermatozoon was considered to be motile when its average path velocity (VAP) was higher than 10 µm·s^{-1}.

2.5. Flow cytometry assessment

Mitochondrial membrane potential, sperm viability and acrosome integrity were evaluated by flow cytometry. Details of flow cytometry experiments are given according to the International Society for Advancement of Cytometry (Lee et al., 2008). The flow cytometer used for this study was a Cell Laboratory Quanta SC™ (Beckman Coulter; Fullerton, CA, USA) and particles were excited with an argon ion laser (excitation: 488 nm; power: 22 mW). Cell diameter and volume were determined by the Coulter principle. The electric volume channel was periodically calibrated with 10-µm flow-check fluorospheres (Beckman Coulter).

Three different optical filters were used with the following characteristics: FL1 (green fluorescence) to detect PNA, SYBR-14 and JC-1 monomers, the detection width was between 505–545 nm; FL2

(orange fluorescence) to detect JC-1 aggregates, the detection width was between 560–590 nm; FL3 (red fluorescence) to detect fluorochromes PI, the detection width was between 655–685 nm.

Sheath flow-rate was set at 8 $\mu L \cdot min^{-1}$. The analyzer threshold was set to exclude cell aggregates (> 12 µm diameter) and subcellular debris (<7 µm diameter). Dot-plots were examined by Cell Lab Quanta SC MPL Analysis Software (version 1.0; Beckman Coulter). Flow cytometry results were corrected on the basis of debris particles determined by the SYBR14/PI test. All samples were evaluated in triplicate.

2.5.1. Sperm viability

Sperm viability was detected by using two fluorescent probes, SYBR-14 and Propidium Iodide (PI), included in the LIVE/DEAD Sperm Viability Kit (Molecular Probes, Invitrogen) (Garner and Johnson, 1995; Huo et al., 2002). Sperm samples were first diluted to a final concentration of 1×10^6 spermatozoa$\cdot mL^{-1}$ with PBS (phosphate-buffered saline) and aliquots of 500 µL were first stained with 0.5 µL SYBR-14 (final concentration: 100 nM) for 10 min at 38ºC in darkness, and then with 2.5 µL PI (final concentration: 12 µM) at the same conditions for 5 min.

Two filters, FL-1 (SYBR-14 detection) and FL-3 (PI detection), were used and the following sperm populations were distinguished: a) viable spermatozoa ($SYBR14^+/PI^-$); b) non-viable spermatozoa ($SYBR14^-/PI^+$ and $SYBR14^+/PI^+$). Debris particles were stained by no fluorochrome ($SYBR14^-/PI^-$).

2.5.2. Acrosome integrity

Acrosome integrity was assessed by staining with a lectin from *Arachis hypogaea* (PNA) conjugated with FITC (fluorescein isothiocyanate) and PI (PNA-FITC/PI test). Sperm samples were diluted to a final concentration of 1×10^6 spermatozoa$\cdot mL^{-1}$ with PBS and aliquots of 500 µL were incubated with 0.5 µL PNA-FITC (final concentration: 1.25 mg/mL) and 2.5 µl PI (final concentration: 12 µM) in darkness for 10 min at 38°C (Pinart et al., 2015). As spermatozoa were not previously permeabilized, they were divided into the following four populations: a) viable spermatozoa with an intact acrosome ($PNA\text{-}FITC^-/PI^-$), spermatozoa with intact plasma membrane and outer acrosome membrane (OAM), unstained; b) spermatozoa with a non-intact plasma membrane ($PNA\text{-}FITC^+/PI^-$); c) non-viable spermatozoa with an intact acrosome ($PNA\text{-}FITC^+/PI^+$); d) non-viable spermatozoa with a non-intact acrosome ($PNA\text{-}FITC^-/PI^+$).

2.5.3. Mitochondrial membrane potential

Mitochondrial membrane potential (MMP) was detected by JC-1 (5,5',6,6'-tetrachloro-1,1',3',3'3 tetraethylbenzimidazolylcarbocyanine iodide) staining. JC-1 allows distinction between spermatozoa with low and high MMP. At low MMP, JC-1 exists as a monomer exhibiting green fluorescence, whereas the stain creates aggregates and changes the fluorescence from green to orange at high MMP (Hossain et al., 2011).

Briefly, sperm samples were first diluted to a final concentration of 1×10^6 spermatozoa·mL^{-1} with PBS and aliquots of 500 µl were incubated with 0.5 µL JC-1 (final concentration: 3µM) at 38 °C in the dark for 30 min (Huo et al., 2002). Sperm cells were divided into two populations, low and high MMP: a) spermatozoa with high MMP mainly emitted at the orange light spectrum and appeared in the upper half of FL1 vs. FL2 dot-plots; b) spermatozoa with low MMP only emitted at the green light spectrum and appeared in the lower half of FL1 vs. FL2 dot-plots.

2.6. Statistical analysis

Results were analyzed with a statistical package (IBM® SPSS® 21.0 for Windows; IBM Corp.; Chicago, IL, USA). Before conducting any test, data were checked for normality (Shapiro-Wilk test) and homogeneity of variances (Levene test). The level of significance was set at $P\leq0.05$ in all cases and, unless stated otherwise, data are shown as mean ± standard error of the mean (SEM).

2.6.1. Classification of ejaculates into groups of good (GFE) and poor freezability (PFE)

Ejaculates were classified into two groups of freezability (i.e. resilience to withstand cryopreservation; GFE and PFE) according to their total sperm motility and membrane integrity ($SYBR14^+/PI^-$) determined at 30 and 180 min post-thaw in control samples. Separate cluster analyses based on the log-likelihood distance and the Bayesian Schwarz criterion were conducted and a number of groups of two was set.

2.6.2. Effects of photo-stimulation on the cryotolerance of GFE and PFE

The effects of photo-stimulation before cryopreservation on the viability, acrosome activity, mitochondrial activity and sperm kinetic parameters in GFE and PFE were evaluated through a linear mixed model followed by a Sidak's post-hoc test. Post-thawing time (30 min and 180 min) was the intrasubject factor and the treatment (control or photo-stimulation) and the freezability group (GFE or PFE) were the fixed-effects factors. There was no random-effects factor, as each ejaculate came

from a separate boar. All sperm parameters were considered as dependent variables. As total sperm motility did not fit with parametric assumptions (i.e. normal distribution and homogeneity of variances), this parameter was transformed with arcsin square- root.

3. Results

3.1. Sperm viability

As expected and due the nature of ejaculate groups, GFE presented a higher percentage of viable spermatozoa (SYBR14$^+$/PI$^-$) than PFE, at both post-thaw incubation times (30 min and 180 min) (Fig. 1). However, the effect of semen photo-stimulation before cryopreservation was different considering the two ejaculate groups.

In the case of GFE, photo-stimulation reduced ($P<0.05$) the percentage of SYBR14$^+$/PI$^-$ spermatozoa when compared to that of the control. When looking into post-thaw incubation, the extent of the decrease in GFE photo-stimulated samples was higher than that of GFE control (Fig. 1). In contrast, photo-stimulation of PFE provided better results. At 180 minutes post-thaw, as shown in Figure 1, sperm viability of PFE was significantly ($P<0.05$) higher than that of the control (20.91% ± 3.35 PFE Photo *vs.* 9.33% ± 2.52 PFE Control) and similar to that of GFE photo-stimulated at the same time.

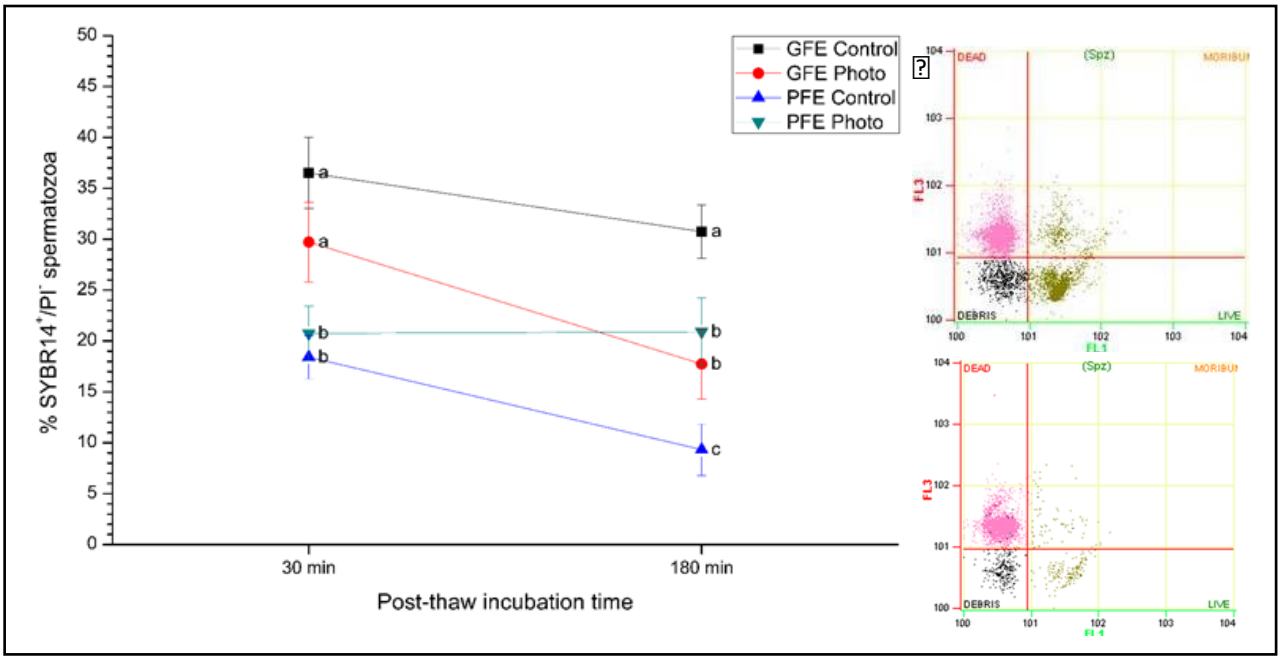

Figure 1. *A) Left side: Percentage of viable spermatozoa (SYBR14+/PI-) at Post-thaw incubation times (30 min and 180 min). The spermatozoa were divided into two principal groups (GFE/PFE) for each treatment (control and photo). Letters show significant ($P < 0.05$) differences when compared*

with the respective counterpart 30 min point. B) Right side: FL1 vs FL3 dot-plots of SYBR14/PI of PFE boar at 180 minutes. The upper dot-plots represents the photo treatment and the lower dot-plots represents the control treatment.

3.2. Acrosome integrity

Again as expected, GFE presented significantly (P<0.05) higher percentages of viable spermatozoa with intact acrosome membrane (PNA-FITC$^-$/PI$^-$) than PFE group at 30 min of evaluation (Fig. 2).

Within GFE, photo-stimulation did not significantly differ from control group at both evaluation times, although acrosome integrity in the control group tended to be higher than that of the photo-stimulated (control vs. photo: 28.25% ± 2.55 vs. 23.53% ± 3.16 at 30 min post-thaw; 21.72% ± 2.80 vs. 17.33% ± 3.05 at 180 min post-thaw).

Conversely, PFE photo-stimulated group significantly (*P*<0.05) improved the sperm quality in relation with this parameter. At two post-thaw incubation times (30 and 180 min) photo-stimulation group maintained their acrosomal integrity better than that of the respective control (control vs. photo: 16.30% ± 1.80 vs. 20.44% ± 1.78 at 30 min post-thaw; 11.95% ± 0.96 vs. 18.74% ± 1.65 at 180 min post-thaw; Fig. 2). At 180 min, the percentage of viable spermatozoa with an intact acrosome did not significantly differ between GFE and PFE in photo-stimulated group.

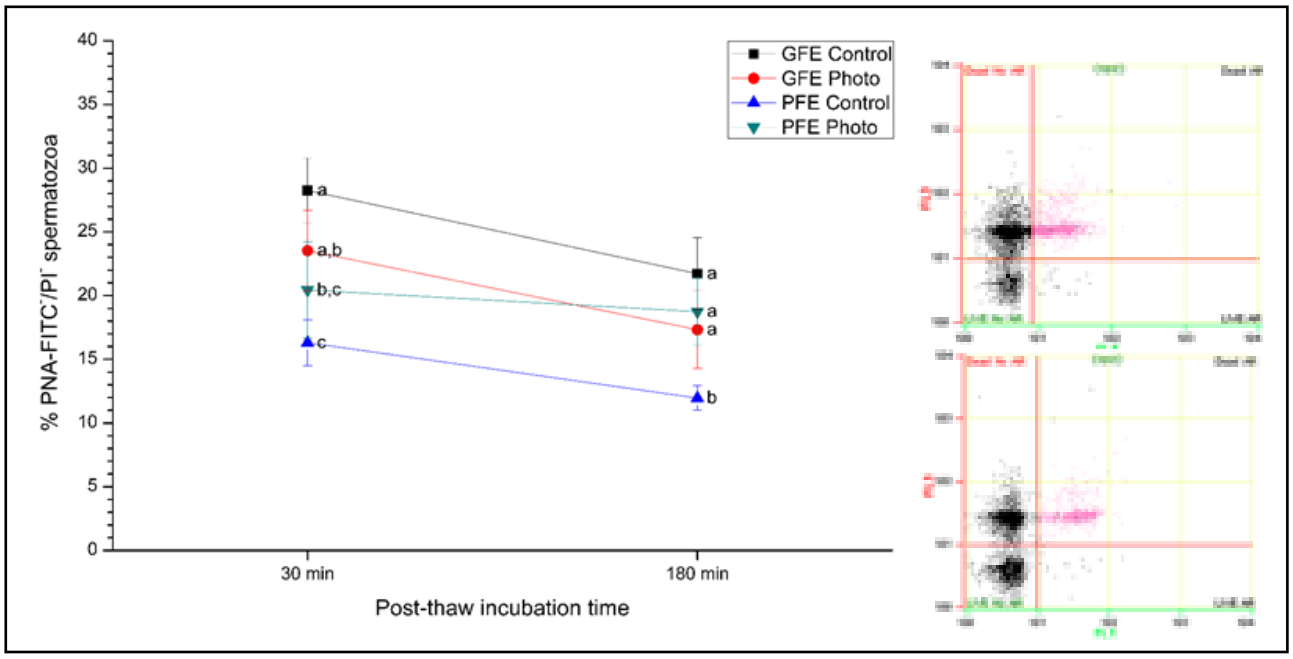

Figure 2. A) Left side: *Percentage of* viable *spermatozoa with intact plasma membrane (PNA-FITC$^-$ /PI$^-$) at post-thaw (30 min and 180 min). Sperm were divided into two principal groups (GFE/PFE) for each treatment (control and photo). Letters show significant (P<0.05) differences when compared*

with the respective counterpart at the same time point. B) Right side: *FL3 vs FL1 dot-plots of PNA-FITC/PI of a PFE ejaculate at 30 minutes. Viable spermatozoa with intact acrosome appear in the lower left of FL1 vs. FL3 dot-plots.*

3.3. Mitochondrial activity

The experimentally results of JC-1 assessment displayed no relevant variations in the percentage of spermatozoa of high mitochondrial activity, both in GFE and PFE. However, photo-stimulation treatment showed interesting results on Geomean values (intensity of JC-1 fluorescence). As reported in Table 1, photo-stimulation of PFE showed a significantly ($P<0.05$) percentage of spermatozoa with high MMP than the control, even at 30 min post-thaw. In contrast, no differences between GFE treatments were found at 30 and 180 min post-thaw.

Table 1. *Geomean values (mean ± SEM) of high mitochondrial membrane potential in GFE and PFE ejaculates. These values were obtained at 30 and 180 minutes post-thaw for control and photo-stimulation treatment. Superscript letters showed significant ($P < 0.05$) differences between treatments and ejaculate groups.*

Ejaculate classification	Treatment	Time (min)	Mitochondrial membrane potential (geomean ± SEM)
GFE	Control	30 min	31.05±3.30 ^{a}
		180 min	28.63±3.65 ^{a}
	Photo	30 min	32.21±2.88 ^{a}
		180 min	33.21±3.96 ^{a}
PFE	Control	30 min	37.45±2.75 ^{a}
		180 min	61.18±2.36 ^{b}
	Photo	30 min	74.83±3.74 ^{c}
		180 min	66.88±2.91 ^{b}

3.4. Sperm motility

Results of general motility are shown at Table 2. As expected, motility parameters were significantly higher in GFE than in PFE. However, in contrast to the other parameters examined in this study, photo-stimulation reduced the motility of both GFE and PFE. The detrimental effects of photo-stimulation on sperm motility were observed both at 30 than 180 min.

***Table 2**. Motility parameters of GFE (good freezability ejaculate) and PFE (poor freezability ejaculate). Boar samples were subjected to two treatments: control and photo-stimulation before cryopreservation. Samples were evaluated at 30 minutes and 180 minutes post-thaw. Superscript letters showed significant (P < 0.05) differences when compared with the respective counterpart at the same time point.*

Ejaculates classification	**GFE**				**PFE**			
Treatment	**Control**		**Photo**		**Control**		**Photo**	
Time (min)	**30 min**	**180 min**	**30 min**	**180 min**	**30 min**	**180 min**	**30 min**	**180 min**
Total motility (%)	30.10±1.49 ^{a}	21.15±3.48 ^{a}	23.45±4.51 ^{a}	9.90±1.79 ^{b}	13.49±1.38 ^{b}	7.41±1.06 ^{b}	11.63±1.79 ^{b}	7.45±0.81 ^{b}
Progressives (%)	22.7±1.74 ^{a}	15.37±3.38 ^{a}	15.26±3.59 ^{a}	4.55±1.76 ^{b}	6.67±1.16 ^{b}	2.23±0.65 ^{b}	5.34±1.56 ^{b}	1.87±0.52 ^{b}
VCL (µm·s^{-1})	67.93±3.34 ^{a}	58.63±4.61 ^{a}	59.51±4.32 ^{b}	41.86±5.38 ^{b}	45.70±3.47 ^{c}	29.03±4.55 ^{c}	43.56±4.30 ^{c}	34.12±4.32 ^{c}
VSL (µm·s^{-1})	52.30±2.96 ^{a}	42.38±4.38 ^{a}	36.23±4.46 ^{b}	25.29±4.84 ^{b}	31.82±3.29 ^{b}	19.16±3.62 ^{b}	25.29±3.68 ^{c}	16.83±3.46 ^{b}
VAP (µm·s^{-1})	59.60±2.93 ^{a}	48.98±4.39 ^{a}	45.01±4.14 ^{b}	31.31±4.95 ^{b}	38.82±3.65 ^{b,c}	22.86±3.94c	32.91±3.80 ^{c}	23.09±3.61 ^{c}
LIN (%)	76.9±2.09 ^{a}	70.41±3.32 ^{a}	59.29±6.16 ^{b}	55.44±7.23 ^{b}	63.53±3.81 ^{b,c}	56.86±4.30 ^{b}	52.93±4.34 ^{c}	47.63±5.32 ^{b}
STR (%)	87.49±1.68 ^{a}	84.80±2.70 ^{a}	75.95±5.69 ^{b,c}	74.04±5.59 ^{b}	78.86±2.80 ^{b}	74.96±3.49 ^{b}	70.32±3.88 ^{c}	66.87±4.28 ^{c}
WOB (%)	87.74±0.76 ^{a}	82.53±1.70 ^{a}	74.86±4.11 ^{b}	71.30±5.22 ^{b}	74.70±3.92 ^{b}	73.25±2.95 ^{b}	72.16±2.93 ^{b}	66.32±3.79 ^{c}
ALH (µm)	2.05±0.07 ^{a}	1.94±0.10 ^{b}	2.11±0.21 ^{a}	1.50±0.20 ^{b,c}	1.82±0.29 ^{a}	1.03±0.17 ^{c}	1.47±0.16 ^{b}	1.34±0.22 ^{b,c}
BCF (Hz)	8.20±0.24 ^{a}	7.92±0.54 ^{a}	6.77±0.82 ^{b}	5.42±1.01 ^{b}	5.84±0.55 ^{b,c}	3.52±0.70 ^{c}	4.46±0.64 ^{c}	2.93±0.62 ^{c}

4. Discussion

Cooling and thawing are the most delicate steps of semen cryopreservation because inflict most of the sperm cryoinjuries (Yeste, 2015). At post-thaw, boar spermatozoa may present nucleoprotein-DNA structural alterations (Flores et al., 2008, 2011), changes in mitochondrial membrane potential (Flores et al., 2010) and capacitation-like changes (Bailey et al., 2000) that can lead to a reduction in fertilizing potential, loss of motility and degenerative acrosome exocytosis (Bravo et al., 2005; Green and Watson, 2001; Bathgate, 2011). Besides, boar sperm is highly sensitive to cold shock due to the high content of unsaturated fatty acids and low presence of cholesterol molecules in plasma membrane (Yeste, 2015). All these damages lead to a reduction in the sperm fertilizing ability and, thus, the use of cryopreserved semen is extremely limited.

Additionally, boar spermatozoa present high variability in their resilience to withstand cryopreservation (also known as freezability). This variability is not only found between individuals but also between ejaculates from the same male. For this reason, boar ejaculates are usually classified as GFE and PFE (Casas et al., 2009; Thurston et al., 2002), based on their post-thaw sperm survival and motility. Unfortunately, at present, it is not possible to predict the ejaculate freezability before cryopreservation takes place (Yeste, 2016). Differences in fatty acid composition and genetic markers have been found to be related with cryotolerance (Thurston et al., 2002; Waterhouse et al., 2006), but mechanistic studies are still required to understand which this link is. While research on this realm is much warranted, it is also important to find applied strategies to support ejaculate cryotolerance. In this way, it may be of interested cryopreserving not only GFE but also PFE, especially if PFE come from highly valuable boars.

As described in the introduction section of this work, previous studies demonstrated that low laser irradiation could have beneficial effects on sperm cells in several animal species (Abdel-Salam and Harith, 2015). Thus, sperm photo-stimulation could be a means to improve cryotolerance for both GFE and PFE. To the best of our knowledge, no research on the effects of photo-stimulation on boar sperm cryotolerance has been conducted thus far. For this reason, the current work aimed at evaluating whether red-light LED photo-stimulation prior to starting freezing procedures could increase the cryotolerance of ejaculates.

Our experimental data indicated an improvement of post-thaw sperm viability in PFE subjected to photo-stimulation procedures. In agreement with this, similar results were reported by (Fernandes et al., 2015) when bull sperm were irradiated before cryopreservation. Other works described better preservation of viable sperm in ram and avian species when photo-stimulation was performed at post-

thaw (Iaffaldano et al., 2013, 2016; Nicolae et al., 2015). If photo-stimulation provided a beneficial effect on boar PFE group, the same cannot be said for GFE where this approach had the opposite effects.

As for the SYBR14/PI test, photo-stimulation showed different results between GFE and PFE on acrosome integrity assessment. Some authors reported that low-laser irradiation had beneficial effects on acrosome integrity (Fernandes et al., 2015; Iaffaldano et al., 2010) but photo-stimulation performed in GFE was detrimental. On the contrary, the percentage of viable spermatozoa with an intact acrosome membrane (PNA-FITC$^-$/PI$^-$) was higher in PFE photo-stimulated group than in PFE control along the entire evaluation time. The concurrence between viability and acrosome integrity results is logical because both SYBR14/PI and PNA-FITC/PI tests analyze the membrane integrity thanks to PI staining and an intact plasma membrane is a common characteristic of PNA-FITC$^-$/PI$^-$ and SYBR14$^+$/PI$^-$ sperm populations. However, the mechanisms that could explain how photo-stimulation could act through a different manner between GFE and PFE groups still unidentified.

The improvement of sperm viability and acrosome integrity was not the only effect reported in the scientific literature on semen photo-stimulation. The most noticeable finding in the use of low-irradiation on spermatozoa cells was an improvement in motility parameters and ATP content (reviewed in Abdel-Salam and Harith, 2015). Furthermore, some authors showed that mitochondrial exposure to laser irradiation could stimulate the activity of cytochrome C/Cytochrome C oxidase (Iaffaldano et al., 2010) and increase calcium uptake rates (Breitbart et al., 1996; Lubart et al., 1992), changing the balance between glycolysis and oxidative phosphorylation (Yeste et al., 2016). Yeste et al. (2016) suggested that red-light LED irradiation could increase mitochondrial activity of boar spermatozoa, as light could interact with compounds of the mitochondrial electronic chain, which is made up of by several enzymatic complexes that are located in the inner mitochondrial membrane. For all these reasons, in our work we also analyzed mitochondrial activity and numerous motility parameters of sperm cells. While JC-1 assessment did not show any variation in the percentage of high mitochondrial membrane potential (MMP) for all ejaculate groups, treatments and evaluation times, the fluorescence intensity (Geomean) of mitochondrial activity provided interesting results. However, once again, photo-stimulation showed improving effects only on PFE, displaying, in this group, higher mitochondrial activity at 30 min post-thaw than respectively control.

Photo-stimulation could also modulate the production of ROS species, as ROS formation is caused by disruption of electron transport. These reactive species are mainly produced by mitochondria and are responsible for acrosome exocytosis (Liu et al., 2002) and activation of apoptotic-like signals linked to sperm capacitation (Begum et al., 2013). Therefore, the marketed effect on mitochondrial

membrane potential is remarkable in the context of the current study and should be further investigated, as ROS formation also disrupts the link between electron transport chain and oxidative phosphorylation.

On the other hand, the results in mitochondrial membrane potential should have an impact on general sperm motility, as motility depends of mitochondrial energy. As mentioned above, some studies reported that semen irradiation improved both frozen-thawed than liquid-storage sperm motility for several animal species (ram, turkey, and rabbit) (Iaffaldano et al., 2010, 2013; Zan-Bar et al., 2005). In contrast with mitochondrial activity outcome, and also with the results of viability and acrosome integrity discussed above, not only we did not see any improvement in photo-stimulated PFE group, but a detrimental effect on sperm general motility was observed in both types of ejaculates (GFE and PFE) (see Table 2). In addition, and as aforementioned, JC-1 assay in PFE showed a higher mitochondrial activity, which should translate into more available energy for the sperm cell, improving motility parameters (Paoli et al., 2011). However, in this case, photo-stimulation showed a controversial effect: the relative high MMP fluorescence was coupled with a reduction of motile sperm population. According to the literature, ejaculates with low motility rate show an unexpectedly high proportion of sperm with high MMP, suggesting that mitochondrial respiration could not be the only support for sperm motility (Volpe et al., 2009).

Thus, further research should be conducted in order to clarify this issue when photo-stimulation is performed.

5. Conclusion

In conclusion, LED photo-stimulation procedures increase the resilience of poor freezability ejaculates (PFE) to withstand cryopreservation, especially when viability, acrosome integrity and mitochondrial activity are evaluated at 180 minutes post-thaw. On the contrary, photo-stimulation had no effect on good freezability ejaculates (GFE). While these results indicate that photo-stimulation may be considered as potential tool to increase the cryotolerance of poor freezability ejaculates, more research on the mechanisms underlying the detrimental effects on sperm motility is required, as this may allow to understand whether such increase in cryotolerance has a significant impact upon the fertilizing ability of PFE.

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GENERAL DISCUSSION

The aim of this thesis was to study two methods for improving semen quality during storage in porcine and equine species: natural antioxidant supplementation and semen photo-stimulation.

As each of the four papers reported in this thesis has its own discussion and conclusion about the results obtained, this chapter will simply consists in a general discussion on issues and perspectives emerged from the researches performed during my experience as PhD student.

Boar sperm cryopreservation is the best technology to store semen for long periods for preserving genetic material through time and for planning AI but cryopreservation induces alterations in spermatozoa that hesitate in a loss of fertilizing ability (Didion *et al.*, 2013). This is mainly due to two important events occurring during freezing and thawing procedures: the excessive ROS generation and the parallel decrease in antioxidants defences. In order to improve post-thaw quality of boar sperm, various antioxidants are routinely added in freezing protocols and new molecules are continuously studied (Yeste, 2016).

There is a wide variety of antioxidants (natural or synthetic) that act with different mechanisms. In fact, these molecules are not only classified by their chemical nature (enzymatic or non-enzymatic) but also considering, for example, how they can reduce ROS presence. Some antioxidants are called "preventive" as, being transition metal chelating agents (e.g. ferritin or haptoglobin), they can prevent ROS formation; others are defined as "scavengers" in that they can reduce ROS amount (e.g. vitamin E), while a further category of enzymes named "repairing agents" is involved when ROS damage already exists and it must be repaired (e.g. polymerases or transferases) (Polimeni and Aperio, 2013). Antioxidants' proprieties and possible mechanisms of action can be evaluated by studying the molecular structure. Polyphenols, like RESV or EGCG, are antioxidant and scavenging agents for definition, thanks to the large number of –OH groups in the molecular structure (Higdon and Frei, 2003). However, when these molecules are used in the practice, the situation becomes more complicated. The knowledge on how antioxidants can act is not enough to figure out what their impact on a cell will be. As product of spermatogenesis, a process in which numerous cellular constituents are lost or modified, mature spermatozoa may seem a "simplified" cell, but it is not like that. Spermatozoa, in order to become competent to fertilize an oocyte, undergo function and structure modifications. These physiological alterations are guided by different metabolic pathways, which are not completely known and in which many types of reactive oxygen and nitrogen species

seem to be involved. Consequently, when a molecule with proved antioxidant properties is used in semen extender supplementation, it is not easy to predict its effect on spermatozoa. Our results of the Paper 1 and 2 reported in this thesis can be an example. Both RESV and EGCG supplementation (alone or in combination) on thawing extender had a positive effect on boar sperm as they significantly stimulated sperm fertilizing ability, increasing oocyte penetration rate, although both antioxidants were washed away before gamete co-incubation. This result suggests that the protective action during thawing can lead to positive effects on sperm function that, in turn, are responsible for the subsequent increased fertilizing ability even if the molecules are not present anymore. The increase in penetration rate could be explained by an increased number of capacitated or hyperactivated spermatozoa.

For this purpose, we studied the changes due to the two natural antioxidants in the localization of sperm tyrosine phosphorylated proteins, parameter known to be related to sperm capacitation (*Paper 2)*. Immunolocalization results clearly show no difference between groups after 1 h incubation in capacitation condition. Evidently, this parameter could be considered too "downstream" in the capacitation events to be thoroughly changed by the different treatments (Bucci *et al.*, 2012b). It should also be remarked that, after 1 h of incubation in BTS medium, no difference was found between groups; in particular, almost 95% of the sperm cells showed the F pattern, and therefore they did not seem to undergo any capacitation.

Moreover, RESV is more powerful than EGCG in enhancing penetration rate (*Paper 2*); in fact, whereas ECGC induced a significant increase in the percentage of fertilized eggs, the extent of that increase was even higher in the case of RESV, which did not show differences with respect to the treatment with both antioxidants (RESV+EGCG).

To better determine whether any change on sperm metabolism and membrane composition could result from the addition of RES and ECGC to frozen-thawed spermatozoa, we studied a wide panel of sperm parameters (*Paper 2)*. However, the presence of antioxidants seems to be ineffective on viability, acrosome integrity, lipid peroxidation and mitochondrial function. Therefore, the mechanism by which these molecules act on spermatozoa is still unclear. Possibly, a sum of little and non-statistically relevant changes in sperm functions could explain the results obtained on *in vitro* fertilization.

Another important issue to kept in mind when using these molecules is that antioxidants can also work as pro-oxidant molecule in certain conditions and/or concentrations. When this phenomenon

occurs, antioxidants can act as reactive species, damaging sperm cell: EGCG was demonstrated to be able to produce hydrogen peroxide (Furukawa *et al.*, 2003) that could damage DNA and sperm plasma membrane via lipid peroxidation (Chen *et al.*, 1997; Kemal Duru *et al.*, 2000; Kusakabe and Kamiguchi, 2004), while high RESV concentrations can increase plasma membrane phospholipid disorder and reduce the percentage of motile and viable spermatozoa (Martín-Hidalgo *et al.*, 2013; Tvrdá *et al.*, 2015b). Therefore, it is necessary to define the best antioxidant concentration in order to maximize its beneficial properties on sperm cells and to avoid, at the same time, adverse effects. For example, in a preliminary experiment of the Paper 3 (data not shown), high concentrations of green tea extract polyphenols resulted to be toxic for stallion sperm. A significant decline in sperm motility and viability was observed, with a 100% dead immotile spermatozoa after 24 h. Finally, the effect of antioxidants may vary depending on species. In fact, for example, an antioxidant can be effective in dog sperm but not in stallion one. Green tea polyphenols could be effective in increasing both motility and viability in dog and ram cooled sperm (Wittayarat *et al.*, 2013; Husam J. H. Banana, 2015), while they are not useful in stallion semen liquid storage (Bucci *et al.*, 2017).

Moreover, the sperm storage technique influences the activity of antioxidant molecules which could be effective in frozen-thawed semen but not in cooled one: in bull sperm, for example, when RESV supplementation is performed before cryopreservation, it could significantly improve frozen-thawed sperm motility, mitochondrial membrane potential and reduce DNA damages (Bucak *et al.*, 2015) while increasing acrosome reaction when supplemented during the liquid storage (Assunção *et al.*, 2015). In addition, RESV has also been reported to be ineffective in improving boar sperm quality during liquid storage (Martín-Hidalgo *et al.*, 2013).

Another feature that can influence porcine sperm storage and cryopreservation, as in other species, is the individual variability of sperm cryotolerance which underlies the existence of good (GFE) and poor (PFE) freezability ejaculates. Unfortunately, at present, it is not possible to predict the ejaculate freezability before that cryopreservation takes place (Yeste, 2016). Differences in fatty acid composition and genetic markers have been found to be related with the cryotolerance (Thurston *et al.*, 2002; Waterhouse *et al.*, 2006), but mechanistic studies are still required to understand which this link is. While research on this realm is much warranted, it is also important to set up strategies to support ejaculate cryotolerance. In this way, it may be interesting the cryopreservation of both GFE and PFE, especially if PFE comes from highly valuable boars.

As already stated, previous studies demonstrated that low laser irradiation could have beneficial effects on sperm cells in several species (Abdel-Salam and Harith, 2015). Thus, sperm photo-stimulation could be useful to improve cryotolerance for both GFE and PFE and that was the goal of the last paper (Paper 4) in which red-light LED photo-stimulation prior to freezing procedures was applied to both GFE and PFE. LED photo-stimulation procedures improved post-thaw sperm viability in PFE but not in GFE where this approach had the opposite effects. Same results were also reported by Fernandes *et al.*, 2015 who irradiated bull sperm before cryopreservation. Other works described better preservation of viable sperm in ram and avian species when photo-stimulation was performed at post-thaw (Iaffaldano *et al.*, 2013, 2016; Nicolae *et al.*, 2015).

As for viability, photo-stimulation increased acrosome integrity and mitochondrial activity of PFE but not GFP group. Moreover, a detrimental effect on sperm general motility was observed in both types of ejaculates.

These results indicate that photo-stimulation may be considered as a potential tool to increase the cryotolerance of poor freezability ejaculates but more research on the mechanisms underlying the detrimental effects on sperm motility is required, as this may allow to understand whether such increase in cryotolerance has a significant impact upon the fertilizing ability of PFE.

CONCLUSIONS

Overall, the results of this thesis demonstrate that the addition of RESV or EGCG, alone or in combination, to thawed boar semen positively affects *in vitro* penetration rate (*Papers 1-2*), while no positive effects were registered in cooled stallion semen up to 48 h storage when EGCG or GT-PFs were added (*Paper 3*). As a consequence, the addition of these substances to cooling medium for stallion sperm storage seems not useful. Instead, the results obtained in boar sperm could be important not only *in vitro* but also *in vivo*. RESV and EGCG can increase the fertilizing ability of thawed boar semen, even if the two molecules are no more present at the moment of oocyte fertilization. This means that a positive effect on sperm function must exist, but any sperm parameters analyzed can help to explain this phenomenon. Further studies are necessary to:

- understand the mechanism/s by which RESV and EGCG act on sperm cells;
- verify whether the zygote development could be affected or not by their presence;
- verify whether the addition of these antioxidants in the commercial thawing medium might enhance sperm fertilizing ability and reproductive performance during porcine AI with frozen-thawed semen.

Photo-stimulation may be considered as another potential tool to increase frozen-thawed boar semen quality (*Paper 4*). This practice could significantly enhance the cryo-tolerance of poor freezability ejaculates and could have enormous profits in the daily use of boar cryopreserved semen. However, also in this case, more researches are required to understand how red LED-based light exerts its action upon sperm cells as well as the mechanism underlying the detrimental effect on sperm motility registered; in addition, it has to be established whether such an increase in boar sperm cryo-tolerance has a significant impact upon the fertilizing ability of poor freezability ejaculates.

As for porcine species, any method studied (antioxidants supplementation and semen photo-stimulation) seems to be effective in improving frozen-thawed semen quality. As already discussed, the practice of antioxidants addition on semen extender is affected by dose-dependency, species-specificity, type of semen storage and sometimes by the variability between ejaculates from the same male.

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