UNIVERSIDAD DE MURCIA

ESCUELA INTERNACIONAL DE DOCTORADO

Role of the Nitric Oxide System in Different Reproductive Processes

Papel del Sistema de Óxido Nítrico en Diferentes Procesos Reproductivos

D. Florentin-Daniel Staicu

2019

The experiments described in this thesis were supported by the Rep-Biotech Joint Doctoral Project, funded by the European Commission under the Horizon 2020 Programme (H2020 MSC-ITN-EJD 675526 REP-BIOTECH) and by the Spanish Ministry of Economy and Competitiveness (MINECO) and the European Regional Development Fund (FEDER), Grants AGL2015–66341-R.

ACKNOWLEDGEMENTS

ACKNOWLEDGEMENTS

At the end of this milestone, I would like to express my gratitude to all the people who accompanied me during this three-year adventure.

A special thanks goes to my supervisors, who made this project possible.

To Carmen, for believing in me since day one and for your priceless help when many of my experiments failed. Thank you for showing me how to sail, I know I still haven't learned how to swim, as promised, but I will sooner or later.

To Juan Carlos, for welcoming me at IVI and teaching me the inner workings of a fertility clinic. I had lots of fun there and I met so many great people in the Andrology and Embryology laboratories, to whom I extend my thanks.

To Jorge, for his infinite patience during the numerous times I knocked on your door when I couldn't wrap my mind around the SAS code. My stay in Boston is one of my favorite memories from this project and this is in part thanks to you and your welcoming colleagues.

None of this would have been possible without my family. Thank you, Mom and Dad, for your constant support during the great and the rough moments (which were quite a few). Thank you for always making sure I had a delicious snack and a full bottle of water during my long sessions at the computer. You always encouraged me to believe in myself and keep moving forward no matter what. All the sweat and hard work put into this thesis is also yours for having my back at any cost. And thank you Maya, for making me laugh and for wanting to destroy my headphones when you catch me working.

To Gaetano, Flavio, Chiara and Alessia, thank you for sharing together laughs and tears. You were always ready to listen and provide a helpful and much needed advice, either from far away or over a drink and amazing food. All of you made everything seem easier and, thanks to you, I have so many beautiful memories that will cherish forever.

To Sole, Hector, Eve and Cris, for introducing me to Murcia and its lifestyle. Thank you for your many pieces of advice shared over tapas and a beer (or two). Hector, if I now know how to do a Western Blot, I owe it to you.

To Anna, Analuce and Jordana, thank you for your support and I'm very grateful for the funny moments spent together. Anna, it was a pleasure sharing with you our office in Boston and thanks for making me understand what great vodka tastes like.

To all the other Rep-Biotech ESRs, I'm very happy we met, chatted and bonded during our meetings. I hope we can continue to do that in the future.

To Bin and Joseph, thank you for the time we spent watching and discussing movies together and, let's not forget, our rainy New Year's Eve adventure. I look forward to trying a proper Texas barbecue.

To all the members of the Department of Animal Physiology, I much appreciate your kind words of advice. Thank you for having your door always opened and for teaching me how to find solutions by looking at problems from different perspectives.

Last but not least, during this period I was lucky to have met and worked with many other people in this department. Ondrej, Marta, Sergio, Gabi, Ernesto, Julieta, Rebeca, Lucia, Jon, Luis, you all are wonderful persons. We entertained each other by sharing jokes, coffees, tostadas, cakes and so much more. Thank you for making this experience unique.

Thank you all for being part of my journey and making it memorable.

To my parents

INDEX

INDEX

SUMMARY/RESUMEN

SUMMARY

Nitric oxide (NO) regulates both physiological and pathological processes in different systems in mammals, including the vascular, nervous, and reproductive system. Its synthesis takes place from a precursor, namely L-Arginine, thanks to the Nitric Oxide Synthase (NOS) family of enzymes. The latter includes two constitutive enzymes, the endothelial and the neuronal NOS (eNOS, nNOS), and one inducible isoform (iNOS). NOS activity depends not only on the availability of its substrate but also on a series of co-factors, like the reduced form of Nicotinamide Adenine Dinucleotide Phosphate, Flavin Mononucleotide, Flavin Adenine Dinucleotide and Tetrahydrobiopterin.

A considerable amount of studies confirmed that gametes and embryos are capable of producing NO. This was mostly evidenced by detecting the expression and/or localization of the above-mentioned isoforms in different species, including porcine, human, murine, bovine, equine and feline. For this purpose, techniques such as immunocytochemistry, immunohistochemistry, Western Blotting or kinetic assays measuring the conversion rates of L-Arginine to L-Citrulline, were employed.

NO modulates the acquisition of sperm fertilization ability at different levels, including motility, chemotaxis, protein phosphorylation, binding to the zona pellucida (ZP) and acrosome reaction. Nevertheless, it has also been shown that although physiological levels of NO are necessary, higher doses are detrimental to sperm functionality. Specifically, normozoospermic samples appear to release a greater amount of NO than astenozoospermic samples. However, when the sperm are exposed to a higher amount of NO than the endogenous production, the opposite effect is observed. Also, the presence or absence of NO influences the tyrosine phosphorylation of sperm proteins and the sperm's ability to bind to the ZP. It has been shown that NOS activity may be increased by a follicular fluid solution enriched in proteins, which also induces the acrosome reaction in the same spermatozoa.

Extensive studies aimed to determined which were the pathways activated by NO in the male gamete. It has been demonstrated that the soluble isoform of Guanylate Cyclase (sGC) can be activated by NO, leading to a rise in the intracellular levels of cyclic guanosine monophosphate (cGMP). cGMP is involved in the activation of the Cyclic Nucleotide-Gated Channels in the sperm tail, which allow the entry of calcium (Ca^{2+}) ions to the cytoplasm during the capacitation process,

thus regulating sperm motility. cGMP can ultimately lead to the serine/threonine phosphorylation of proteins that promote sperm capacitation and the acrosome reaction through the activation of the cGMP-dependent Protein Kinase (PKG). Additionally, this pathway can also inhibit the degradation of cyclic adenosine monophosphate (cAMP) via cyclic nucleotide phosphodiesterase (PDE). This translates into the activation of the cAMP-dependent Protein Kinase (PKA) and tyrosine phosphorylation. The latter event can be directly regulated by NO via the Adenylate Cyclase (AC) and the Extracellular Signal Regulated Kinase (ERK) pathways. NO can also induce a selective and reversible post-translational protein modification in spermatozoa, named S-nitrosylation. This process consists in the covalent incorporation of NO into thiol groups to form S-nitrosothiols. The function of the nitrosylated proteins is not clear in many cases, but some of them are associated with sperm motility, energy generation and signal transduction.

NOS isoforms have been also identified in oocytes, cumulus cells and in the oviduct, through immunohistochemistry, Western Blotting or quantitative RT-PCR. In the female gamete, NO modulates the meiotic resumption, can prevent oocyte aging and improve the integrity of the microtubular spindle apparatus in aged oocytes. Particularly, NO seems to be of great importance during the oocyte maturation process. In fact, it has been shown that the eNOS-knockout in mice, combined with the inhibition of NO synthesis, results in a low ability of oocytes to reach maturity. Furthermore, NOS inhibitors can decrease the cumulus expansion during *in vitro* maturation, a step that involves protein S-nitrosylation.

In the oviduct, hormones regulate NOS activity during the oestrous cycle. The basal release of NO modulates the oviduct contraction and the beating of the ciliated epithelial cells. Besides, a strict regulation of NO levels is also important for a correct embryo development and implantation. It has been reported that NOS activity varies between the two-cell embryo up to the blastocyst stage. Also, NOS have been described in the endometrium, where NO could contribute to the myometrial relaxation during pregnancy. Human placentas also showed immunoreactivity for NOS isoforms, suggesting a possible role for NO in modulating the placental blood flow and limit platelet aggregation at the interface between maternal and fetal circulations.

Of particular interest in several studies was the attempt to discover new biochemical markers that affect both oocyte and embryo quality and that may be helpful to predict the outcome of *in vitro* fertilization (IVF) techniques. In this sense, to predict the quality of the oocyte, the amount of NO has been determined in the environment where this gamete develops, that is, the follicular fluid (FF). However, the measurement of this gas is complex due to its short half-life, therefore, the determination of nitrite (NO_2) and nitrate (NO_3) is preferred since both species are stable products of

NO oxidation. Even though many techniques can detect these ions, each has its own limitations, which in turn leads to contradictory results in the scientific literature. For instance, an inverse association has been suggested between the NO levels in the FF and the fertilization rate of mature oocytes and embryo morphology. However, other studies did not report similar results, which leads us to reconsider the methodology used to evaluate the amount of NO present in this fluid.

Although a lot of progress has been made to understand the molecular mechanisms that govern the fertilization in mammals, many aspects are still not clear. Particularly, we aimed to better understand the role of NO in this process by analyzing how the addition of NO or its inhibition affected sperm capacitation and IVF in the porcine species (**Chapter 1**), how protein phosphorylation is modulated by NO, L-Arginine and FF during human sperm capacitation (**Chapter 2**) and, finally, how FF NO levels relate to the quality of oocytes in healthy donors (**Chapter 3**).

In the **first chapter** of this thesis, the effect of NO on sperm capacitation and IVF in the porcine species was determined. For this, a NO donor, S-Nitrosoglutathione (GSNO), and two NOS inhibitors, N^G-Nitro-L-Arginine Methyl Ester Hydrochloride (L-NAME) and Aminoguanidine Hemisulfate salt (AG) were used.

Spermatozoa were capacitated in the presence or not of these supplements, after which the sperm capacitation status was evaluated by means of different assays: indirect immunofluorescence, motility assay, Western Blotting, acrosome reaction, phosphatidylserine translocation and measurement of the intracellular Ca^{2+} concentration. Both eNOS and nNOS were located in the sperm head region, with a faint signal in the principal and end piece of the tail. On the other hand, iNOS showed a more general distribution, the immunofluorescent staining being spread over the acrosomal, postacrosomal and neck region, but also in the principal and end piece of the tail. Regarding the motility, phospho-PKA substrates, tyrosine phosphorylation, acrosome reaction, phosphatidylserine translocation and intracellular Ca^{2+} concentration, we observed that they were affected depending on the presence or absence of NO. At time 0, the used treatments did not affect motility patterns, however, after 30 minutes of incubation the straight-line (VSL) and average path velocity (VAP) decreased in the presence of AG. The inhibition of NOS lowered the phosphorylation degree of three PKA substrates (~ 75, ~ 55 and ~ 50 kDa), but tyrosine phosphorylation levels did not differ between the treatments. The inhibitor L-NAME decreased the percentage of acrosome-reacted sperm and phosphatidylserine translocation, whereas both L-NAME and AG reduced the sperm intracellular Ca^{2+} concentration.

Secondly, the role of NO on the interaction of gametes was evaluated in the presence or absence of cumulus cells and with two concentrations of donor and inhibitors. Both female and male gametes were not treated with the NO donor or NOS inhibitors before performing the IVF. The addition of

both L-NAME and AG during IVF induced a decrease in the percentage of penetrated oocytes, when the latter were surrounded by the cumulus cells. This effect was observed with the two concentrations of donor and inhibitors used. Nonetheless, when the cumulus cells were removed, the percentage of penetration was further diminished, becoming null in presence of the inhibitor L-NAME.

In **Chapter 2**, the role of L-Arginine and FF was analyzed on the phosphorylation of serine, threonine and tyrosine residues in capacitated human spermatozoa and whether their effect was modified by the presence of a donor and inhibitors of NO synthesis. For this purpose, once extracted, the sperm proteins were subjected to electrophoresis followed by Western Blotting. We observed that four protein bands of ~ 110, ~ 87, ~ 75 and ~ 62 kDa were affected by the inhibition of NOS in the presence or absence of L-Arginine and FF. Next, these bands were isolated and subjected to in-gel trypsin digestion, followed by mass spectrometry analysis to identify the affected proteins. It was observed that 29 of them were related to different reproductive processes: spermatogenesis, ZP binding, energy and metabolism, stress response, motility and structural organization, signaling and protein turnover.

In our final study (**Chapter 3**), we determined the relationship between the FF levels of NO_2 and NO_3 and the oocyte quality in women participating in a gamete donation program. For each participant, the data regarding the donation cycle and their lifestyle were registered. Moreover, FF samples were collected and processed after oocyte retrieval, to allow the detection of NO_2 and NO_3 via HPLC-UV/VIS. When analyzing the UV chromatograms from standard solutions and FF samples, two peaks were identified and quantified: one located at 10.2 min (identified as NO_2) and one located at 31.6 min (identified as NO_3). Multivariable mixed Poisson and logistic regression models were used to compare total and MII oocyte yields, as well as the proportion of MII oocytes, across tertiles of NO_2, NO_3, total NO and NO_3/NO_2 ratio. The multivariable models were adjusted for age, body mass index, sleep time, coffee intake, smoking history and leisure physical activity, as potential confounders of the relation between NO metabolites and ovarian response to hyperstimulation. Neither total nor MII oocyte yields were associated with the FF concentrations of NO_2 and NO_3. However, the proportion of MII oocytes was directly related to NO_2 levels and inversely to NO_3 levels. No correlation was observed between total NO and the NO_3/NO_2 ratio when compared to the total and mature oocyte yield, nor with the proportion of MII oocytes.

The results obtained in this doctoral thesis show the importance of NO on gametes and their interaction. In relation to porcine spermatozoa, we observed that the location of the NOS isoforms is different and that the inhibition of NO synthesis decreases the parameters involved in the capacitation process and the penetration of oocytes. As far as the human spermatozoa are concerned, we

determined that the inhibition of NO synthesis leads to a decrease in the phosphorylation of proteins related to reproductive functions and, this effect is not reverted by the presence of L-Arginine or FF. In addition, the synthesis of NO in the FF of oocyte donors undergoing superovulation treatment is associated to the proportion of mature oocytes, but not to the total number of oocytes recovered after the treatment.

RESUMEN

El óxido nítrico (NO) regula los procesos fisiológicos y patológicos en diferentes sistemas en mamíferos, incluido el sistema vascular, nervioso y reproductor. Su síntesis tiene lugar a partir del precursor L-Arginina, gracias a la familia de enzimas de óxido nítrico sintasa (NOS). Esta familia se encuentra formada por dos enzimas constitutivas, la NOS endotelial y la neuronal (eNOS, nNOS), y una inducible (iNOS). La actividad de las NOS depende no solo de la disponibilidad de su sustrato, sino también de una serie de cofactores, como la forma reducida de fosfato de dinucleótido de adenina nicotinamida, mononucleótido de flavina, dinucleótido de adenina de flavina y tetrahidrobiopterina.

Se han desarrollado numerosos estudios en los que se confirmó que los gametos y los embriones son capaces de producir NO. Esto se evidenció principalmente al detectar la expresión y/o localización de las isoformas mencionadas anteriormente en diferentes especies, incluyendo porcino, humano, murino, bovino, equino y felino. Para este propósito, se emplearon técnicas tales como inmunocitoquímica, inmunohistoquímica, Western Blotting o ensayos cinéticos que miden las tasas de conversión de L-Arginina a L-Citrulina.

El NO modula la adquisición de la capacidad fecundante de los espermatozoides a diferentes niveles, incluida la motilidad, la quimiotaxis, la fosforilación de proteínas, la unión a la zona pelúcida (ZP) y la reacción acrosomica. No obstante, también ha sido demostrado que, aunque los niveles fisiológicos de NO son necesarios, dosis por encima de estos niveles son perjudiciales para la funcionalidad espermática. En concreto, las muestras normozoospérmicas parecen liberar una mayor cantidad de NO que las muestras astenozoospérmicas. Sin embargo, cuando los espermatozoides se exponen a una cantidad superior de NO a la producción endógena se observa el efecto contrario. Además, la presencia o ausencia de NO influye en la fosforilación en tirosina de las proteínas espermaticas y en la capacidad de los espermatozoides para unirse a la ZP. Se ha demostrado que la actividad NOS puede incrementarse con una solución de líquido folicular enriquecida en proteínas, que también induce la reacción acrosomica en los mismos espermatozoides.

Se han realizado exhaustivos estudios para determinar cuáles eran las vías activadas por el NO en el gameto masculino. Se ha demostrado que la isoforma soluble de guanilato ciclasa (sGC) puede ser activada por el NO, lo que lleva a un aumento en los niveles intracelulares de monofosfato de guanosina cíclica (cGMP). El cGMP participa en la activación de los canales activados por

nucleótidos cíclicos en el flagelo espermático, lo cual permite la entrada de iones de calcio (Ca^{2+}) al citoplasma durante el proceso de capacitación, regulando de esta forma la motilidad. Por otro lado, el cGMP puede conducir en última instancia a la fosforilación en serina/treonina de las proteínas que promueven la capacitación de los espermatozoides y, finalmente la reacción acrosomica a través de la activación de la proteína quinasa dependiente de GMP (PKG). Además, esta vía también puede inhibir la degradación del monofosfato de adenosina cíclico (cAMP) a través de la fosfodiesterasa de nucleótidos cíclicos (PDE). Esto se traduce en la activación de la proteína quinasa dependiente de cAMP (PKA) y fosforilación en tirosina. Este último evento puede ser regulado directamente por el NO a través de la vía de la Adenilato Ciclasa (AC) y la vía de la Quinasa Regulada por Señales Extracelulares (ERK). El NO también puede inducir una modificación postraduccional selectiva y reversible de las proteínas en los espermatozoides, llamada S-nitrosilación. Este proceso consiste en la incorporación covalente de NO en grupos tiol para formar S-nitrosotioles. La función de las proteínas nitrosiladas no está clara en muchos casos, pero algunas de ellas están asociadas con la motilidad de los espermatozoides, la generación de energía y la transducción de señales.

Las NOS también se han identificado en ovocitos, células del cúmulo y en el oviducto, mediante inmunohistoquímica, Western Blotting o RT-PCR cuantitativa. En el gameto femenino, el NO modula la reanudación meiótica, puede prevenir el envejecimiento ovocitario y mejorar la integridad del aparato del huso microtubular en ovocitos envejecidos. En particular, el NO parece ser de gran importancia durante el proceso de maduración de los ovocitos. De hecho, se ha demostrado que la eliminación de la eNOS en ratones, combinada con la inhibición de la síntesis de NO, produce una baja capacidad de los ovocitos para alcanzar la madurez. Además, los inhibidores de la NOS pueden disminuir la expansión del cúmulo durante la maduración *in vitro*, un paso que involucra la S-nitrosilación de proteínas.

En el oviducto, las hormonas regulan la actividad de las NOS durante el ciclo estral. La liberación basal de NO modula la contracción del oviducto y el latido de las células epiteliales ciliadas. Además, una regulación estricta de los niveles de NO también es importante para un correcto desarrollo e implantación del embrión. Se ha visto que la actividad de las NOS varía en el embrión entre el estado de dos células hasta el blastocisto. Además, las NOS han sido descritas en el endometrio, donde el NO podría contribuir a la relajación del miometrio durante la gestación. La placenta también ha mostrado inmunorreactividad para las isoformas NOS, lo que sugiere un posible papel para el NO en la modulación del flujo sanguíneo placentario y limitación de la agregación plaquetaria en la interfaz entre las circulaciones materna y fetal.

De particular interés ha sido el intento de descubrir nuevos marcadores bioquímicos que afectan la calidad de los ovocitos y embriones y que pueden ser útiles para predecir el resultado de las técnicas de fecundación *in vitro* (FIV). En este sentido, para predecir la calidad del ovocito, la cantidad de NO se ha determinado en el entorno donde se desarrolla este gameto, es decir, el líquido folicular (FF). Sin embargo, la medición de este gas es compleja debido a su corta vida media por lo que es necesario recurrir a la determinación de nitrito (NO_2) y nitrato (NO_3) ya que ambas especies son productos estables de oxidación de NO. Son muchas las técnicas que pueden detectar estos iones, no obstante, cada una de ellas tiene sus propias limitaciones, lo cual nos conduce a resultados contradictorios en la literatura científica. Por ejemplo, se ha sugerido una asociación inversa entre los niveles de NO en el FF, la tasa de fecundación y la morfología del embrión. Sin embargo otros estudios no observaron ningún tipo de relación los cual lleva a pensar en la metodología para evaluar la cantidad de NO presente.

A pesar de que se ha avanzado mucho para comprender los mecanismos moleculares implicados en la fecundación en mamíferos, muchos aspectos aún están por ser aclarados. En particular, intentamos comprender mejor el papel del NO en el proceso de fecundación. Para ello, se analizó cómo la adición de NO o la inhibición de su síntesis afecta la capacitación espermática y la FIV en la especie porcina (**Capítulo 1**), cómo la fosforilación de las proteínas es modulada por el NO, L-Arginina y el FF durante la capacitación de espermatozoides humanos (**Capítulo 2**) y, finalmente, cómo los niveles de NO en el FF se relacionan con la calidad de los ovocitos obtenidos en mujeres donantes sanas (**Capítulo 3**).

En el **primer capítulo** de esta tesis se determina el efecto del NO sobre la capacitación de espermatozoides y FIV en la especie porcina. Para ello, se utilizó un donante de NO, S-Nitrosoglutatión (GSNO), y dos inhibidores de las NOS, N^G-Nitro-L-Arginina Metil Éster (L-NAME) y Aminoguanidina (AG).

Los espermatozoides se sometieron a un proceso de capacitación en presencia o no de estos suplementos, después de lo cual se evaluó el estado de la capacitación mediante diferentes ensayos: inmunofluorescencia indirecta, ensayo de motilidad, Western Blotting, reacción acrosomica, translocación de fosfatidilserina y medición de la concentración intracelular de Ca^{2+}. En relación a la localización de las NOS, se observó que tanto la eNOS como la nNOS se ubicaron en la región de la cabeza espermática, con una señal débil en la pieza principal y final de la cola. Sin embargo, la iNOS mostró una distribución más general, extendiéndose la tinción inmunofluorescente sobre la región del acrosoma, postacrosomal y cuello, aunque también en la pieza principal y final de la cola. En cuanto al resto de parámetros evaluados: motilidad, sustratos de fosfo-PKA, fosforilación en tirosina,

reacción acrosomal, translocación de fosfatidilserina y concentración del Ca^{2+} intracelular, observamos que se vieron afectados por la presencia o ausencia de NO. Los parámetros de motilidad a tiempo 0 no experimentaron modificación alguna por presencia/ausencia de NO, sin embargo, a las 30 minutos se observó que la velocidad rectilínea (VSL) y la velocidad media (VAP) disminuyen en presencia de AG. La inhibición de NOS disminuyó el grado de fosforilación de tres sustratos de la PKA (~ 75, ~ 55 y ~ 50 kDa) pero los niveles de fosforilación de tirosina no difirieron entre los tratamientos. El inhibidor L-NAME, hizo disminuir el porcentaje de espermatozoides reaccionados y la exteriorización de la fosfatidilserina. En relación a la concentración de Ca^{2+} intracelular, se observó que tanto L-NAME y AG no permitían el incremento de éste catión durante la incubación en el medio de capacitación.

En segundo lugar, se evaluó el papel del NO sobre la interacción de los gametos en presencia o ausencia de células del cúmulo y con dos concentraciones de donante e inhibidores. Tanto los gametos femeninos como los masculinos no fueron tratados con el donante de NO ni con los inhibidores de las NOS antes de realizar la FIV. Se observó que tanto la adición del L-NAME como del AG durante la FIV, producía un descenso en el porcentaje de ovocitos penetrados cuando éstos se encontraban rodeados por las células del cumulus. Este efecto se observó con las dos concentraciones de donante e inhibidores utilizadas. No obstante, al eliminar éstas células la penetración disminuyó aún más, llegando a ser nula en presencia del inhibidor L-NAME.

En el **Capítulo 2**, se analizó el papel de la L-Arginina y del FF sobre la fosforilación en serina, treonina y tirosina en espermatozoides humanos y si su efecto se veía modificado por la presencia de donante e inhibidores de síntesis de NO. Para este propósito, una vez extraídas las proteínas espermáticas, se sometieron a electroforesis seguida de Western Blotting. Observamos que cuatro bandas de proteínas de ~ 110, ~ 87, ~ 75 y ~ 62 kDa se veían afectadas por la inhibición de las NOS tanto en presencia como en ausencia de L-Arginina o FF. A continuación, estas bandas se aislaron y fueron sometidas a digestión con tripsina en gel, seguido de un análisis de espectrometría de masas para identificar las proteínas afectadas. Se observó que 29 de ellas estaban relacionados con diferentes procesos reproductivos: espermatogénesis, unión a la ZP, metabolismo energético, respuesta al estrés, la motilidad y estructura, y señalización y recambio de proteínas.

En el estudio final (**Capítulo 3**), se determinó la relación entre el nivel de nitrito (NO_2) y nitrato (NO_3) en el FF y la calidad de los ovocitos en mujeres que participaron en un programa de donación de gametos. Para cada donante, se registraron los datos sobre el ciclo de donación y el proprio estilo de vida. Además, las muestras de FF se recolectaron y procesaron después de recuperar los ovocitos, para la detección de NO_2 y NO_3 a través de HPLC-UV/VIS. Al analizar los cromatogramas de UV

de las soluciones estándar y las muestras de FF, se identificaron y cuantificaron dos picos: uno ubicado en 10.2 minutos (identificado como NO_2) y uno ubicado en 31.6 minutos (identificado como NO_3). Para el análisis estadístico, se utilizaron modelos mixtos multivariables de Poisson y regresión logística que permiten comparar número total de ovocitos y de ovocitos en estadio de metafase II, así como la proporción de ovocitos en metafase II, a través de terciles de NO_2, NO_3, NO total y la razón NO_3/NO_2. Como factores de confusión potenciales de la relación entre los metabolitos de NO y la respuesta ovárica a la hiperestimulación, los modelos multivariables se ajustaron según la edad, el índice de masa corporal, el tiempo de sueño, la ingesta de café, el historial de fumo y la actividad física de ocio. Los resultados mostraron que ni el número total de ovocitos recogidos ni el de ovocitos en metafase II se asociaron con los niveles de NO_2 y NO_3 presentes en el FF. Sin embargo, la proporción de ovocitos en metafase II, sí que se relacionó directamente con los niveles de NO_2 e inversamente con los niveles de NO_3. No se observó ninguna correlación entre el NO total y la razón NO_3/NO_2 cuando se compararón con el número total de ovocitos, ovocitos maduros, ni con la proporción de ovocitos en metafase II.

Los resultados obtenidos en la presente tesis doctoral muestran la importancia del NO sobre los gametos y su interacción. En relación al espermatozoide porcino, se observa que la localización de las isoformas de la NOS es diferente y que la inhibición de la síntesis de NO disminuye los parámetros que participan en el proceso de capacitación y la penetración de ovocitos. En relación al espermatozoide humano, queda determinado que al inhibir la producción de NO se produce una disminución de la fosforilación de proteínas relacionadas con la función reproductiva y este efecto no se revierte por la presencia de L-Arginina o FF. Además, la síntesis de NO en el FF de mujeres donantes de ovocitos sometidas a un tratamiento de superovulación, se relaciona con el porcentaje de ovocitos maduros, pero no con la cantidad total de ovocitos recuperados tras el tratamiento.

LITERATURE REVIEW

LITERATURE REVIEW

The following paragraphs that are written in *italics* belong to a book chapter, previously published by Florentin-Daniel Staicu and Prof. Carmen Matas Parra, which is referenced bellow:

Staicu F-D, Matas Parra C. Nitric Oxide: Key Features in Spermatozoa. In: Nitric Oxide Synthase - Simple Enzyme-Complex Roles, Seyed Soheil Saeedi Saravi, InTechOpen; 2017, p. 137–54. doi:10.5772/67551.

The publisher, InTechOpen, authorized its use for this doctoral thesis.

1. INTRODUCTION

The prevalence of infertility is increasing, especially in recent decades. It is estimated that up to 15% of couples in reproductive age are affected by this condition worldwide [1]. Even though its frequency and etiology vary, almost 40% of infertility-related problems can be attributed to a male factor, another 40% to a female factor, and 20% to male and female factors combined [2]. Oxidative stress, due to an augmented synthesis of free radicals, such as the reactive oxygen and nitrogen species (ROS and RNS), represents one of the causes of infertility, particularly in men [3]. Several studies associated both exogenous and endogenous ROS/RNS with decreased sperm motility, abnormal morphology and decreased oocyte penetration (reviewed by Buzadzic *et al*. [2]). In some cases, however, the intake of antioxidants in humans improves reproductive functions [4,5].

It has been shown that ROS/RNS, including hydrogen peroxide, superoxide anion and NO, are essential for the sperm to acquire their fertilizing ability under tightly regulated physiological conditions. *Particularly, NO is a small hydrophobic molecule which can easily diffuse through biological membranes [6]. In vivo, it is synthesized during the conversion of L-Arginine to L-Citrulline by the NOS family of enzymes, with the help of co-factors such as the reduced form of Nicotinamide Adenine Dinucleotide Phosphate, Flavin Mononucleotide, Flavin Adenine Dinucleotide and Tetrahydrobiopterin [7].*

NOS may be found in three different isoforms. Two of them, the endothelial and the neuronal NOS (eNOS, nNOS), require Ca^{2+}/calmodulin to be activated and are responsible for the continuous basal release of NO. The third isoform, known as inducible NOS (iNOS), is Ca^{2+}-independent [8,9].

Since NOS activity depends on the availability of its substrate and its co-factors, all these elements jointly determine the cellular rates of NO synthesis [10].

Spermatozoa produce NO, but this endogenous synthesis might not be sufficient to be physiologically significant [11]. Considerable levels of NO are generated in the female reproductive tract in mammals, which can then lead to the S-nitrosylation of sperm proteins. Consequently, *in vivo*, spermatozoa most likely respond to the exogenous NO produced in the female tract, rather than to the endogenous NO acting as an autocrine signal [11]. In addition, since sex hormones can regulate NO synthesis [12], the levels of this RNS vary during the oestrus cycle [13], thus regulating the fertilization process.

It should be noted that the presence of NO was described in different biological fluids, including the FF. The latter, composed mainly of hormones, enzymes, anticoagulants, electrolytes, ROS and antioxidants, acts as an important mediator for the development of the oocyte [14]. Different reports attempted to determine if the FF components might be useful biochemical markers to predict oocyte and embryo quality and other downstream IVF parameters, such as the pregnancy outcome in couples undergoing infertility treatment [15–20]. When NO was investigated as a potential biomarker, the evidences reported contrasting results. Some works found no associations with oocyte and embryo quality [18,20], whereas others suggested an inverse correlation with the fertilization of mature oocytes and the ability of the subsequent embryo to cleave normally [15]. A clear trend has not yet been defined even when analyzing the association between FF-NO and pregnancy outcome [18,20,21]. Therefore, the variable FF-NO levels could affect the fertilization process *in vivo* and are of particular interest for further comprehensive *in vitro* studies.

The following sections provide a brief overview of the current literature regarding the role of NO in gamete and embryo physiology in mammals.

2. NOS/NO DUO IN SPERMATOZOA

2.1. NOS expression

Numerous studies have been conducted to determine the presence and localization of NOS in sperm from several species (Table 1). For example, Herrero et al. [22] located nNOS in the head of freshly ejaculated human spermatozoa, with a more concentrated fluorescent staining towards the equatorial region. O'Bryan et al. [23] described the pattern of eNOS expression in human spermatozoa, finding that morphologically normal spermatozoa exhibited post-acrosomal and equatorial eNOS immunostaining. Interestingly, though, abnormally shaped sperm cells exhibited

aberrant staining, especially in the midpiece and/or head region, which correlated negatively with the percentage of motile sperm.

NOS were revealed in mature mouse spermatozoa by means of biochemical techniques and Western blot (WB). Herrero et al. [24] showed that mouse spermatozoa can synthesize L-citrulline, depending on the concentration of L-Arginine present in the incubation medium, while different concentrations of N(G)-nitro-L-Arginine methyl ester (L-NAME) inhibits the formation of the amino acid. Furthermore, when sperm protein extracts were incubated under denaturing and non-reducing conditions, and then subjected to immunoblotting assay, a protein fraction of 140 kDa was recognized by the three anti-NOS antibodies.

Bull spermatozoa were examined for the presence of constitutive NOS [25]. NO generation seemed to be enhanced by L-Arginine and abolished by the NOS-inhibitor, L-NAME. In addition, Meiser and Schulz [25] verified the presence of NOS in bull sperm cells by immunohistochemistry, which was confirmed by Western blot. Confocal laser microscopy localized nNOS-related immunofluorescence at the acrosome cap and the main part of the flagellum. The same technique also identified eNOS staining spread over the spermatozoan head. Moreover, when these findings were confirmed by Western blot, immunoreactive bands at 161 kDa (nNOS) and 133 kDa (eNOS) were identified.

Hou et al. [26] investigated whether boar sperm can generate NO, finding that porcine spermatozoa synthesized low levels of NO under non-capacitating conditions, but that the NO concentration almost doubled when sperm were capacitated. Furthermore, NO production was significantly inhibited when capacitated sperm were treated with L-NAME. In another study [27], Western blot analysis was performed to identify NOS enzymes in boar sperm samples. The immunoblots showed three distinct bands: ~ 160 kDa, ~ 130 kDa and ~ 135 kDa, corresponding to nNOS, iNOS and eNOS respectively.

NO production was evaluated in stallion spermatozoa before and after freezing/thawing [28] by means of flow cytometry, after loading the sperm suspension with a NO detection probe. NO synthesis was positively correlated with sperm motility after thawing and, interestingly, the presence of egg yolk in the semen extender radically reduced the amount of NO produced. The authors further investigated in fresh and frozen/thawed stallion sperm the presence of NOS enzymes by WB, using anti-nNOS, anti-eNOS and anti-universal NOS antibodies. Two bands of approximately 83 kDa and 96 kDa were labeled by the antibodies anti-nNOS and anti-eNOS, respectively. Moreover, the other antibody, which recognized an epitope present in all the NOS isoforms described so far, showed two similar bands of 84 and 92 kDa.

Liman and Alan [29], investigated the localization of NOS isoforms in spermatozoa within the intratesticular and excurrent duct systems of adult domestic cats. Overall, the spermatozoa head did not exhibit immunoreactivity. On the other hand, immunoreactivity for all three isoforms was observed in the flagellum, in the proximal cytoplasmic droplets of spermatozoa (located in the neck region) within the lumen of the intratesticular and efferent ducts, in the epididymal duct of the caput epididymis, and in the distal cytoplasmic droplets of spermatozoa (located at the mid-principal piece junction of the tail) within the lumen of corpus and cauda epididymis and the vas deferens.

***Table 1**. Summary of in vitro studies and the techniques used to identify NOS isoforms in different species.*

Species	***Authors***	***Techniques***	***Identified isoforms***
Human	*Herrero et al. [22]*	*Immunofluorescence*	*nNOS*
	O'Bryan et al. [23]	*Immunocytochemistry*	*eNOS*
Mouse	*Herrero et al. [24]*	*Kinetic assays measuring the conversion of L-Arginine to L-Citrulline* *Western blot*	*nNOS, iNOS, eNOS*
Bull	*Meiser and Schulz [25]*	*Modified Griess reaction [30]* *Western blot* *Immunofluorescence*	*nNOS, eNOS*
Boar	*Hou et al. [26]*	*NO assay kit with the Griess reagent*	*nNOS, iNOS, eNOS*
	Aquila et al. [27]	*Western blot*	
Stallion	*Ortega Ferrusola et al. [28]*	*Flow cytometry* *Western blot*	*nNOS, eNOS*
Cat	*Liman and Alan [29]*	*Histochemistry* *Immunohistochemistry* *Western blot*	*nNOS, iNOS, eNOS*

2.2. Role of NO on sperm functionality

Several in vitro studies were conducted in order to determine the effects that NO has on sperm physiology (Fig. 1). Briefly, it has been shown that NO affects sperm motility [23,31,32], acts as chemoattractant [33,34], regulates the tyrosine phosphorylation of different sperm proteins [35,36], enhances the sperm-zona pellucida binding ability [37], and modulates the acrosome reaction [38,39].

Research was first concentrated on determining the effects of NO-releasing compounds on sperm motility and viability. Low concentrations of sodium nitroprusside (SNP), a NO-releasing compound, stimulated sperm hyperactivation in mouse, fish and hamster [40–42], and were beneficial to the maintenance of post-thaw human sperm motility [43]. On the other hand, high concentrations of NO-releasing compounds decreased sperm motility [40,44–46].

Other studies also demonstrated that NO seems to play an important role in the maintenance of sperm motility at physiological levels. A study [32] showed that the basal release of NO by

spermatozoa from normozoospermic samples tended to be greater than that from asthenozoospermic samples, suggesting a physiological and beneficial role for endogenous NO in the preservation of sperm motility. These observations agree with a previous report that normozoospermic spermatozoa express more NOS and generate more nitrite than asthenozoospermic spermatozoa [31]. On the other hand, as previously mentioned, it has been shown that spermatozoa with an abnormal morphology show aberrant staining for eNOS, which was negatively correlated with the motility [23]. A detrimental effect on motility has also been reported by Rosselli et al. [44] and Weinberg et al. [45] when millimolar concentrations of exogenous NO donors were added to sperm samples.

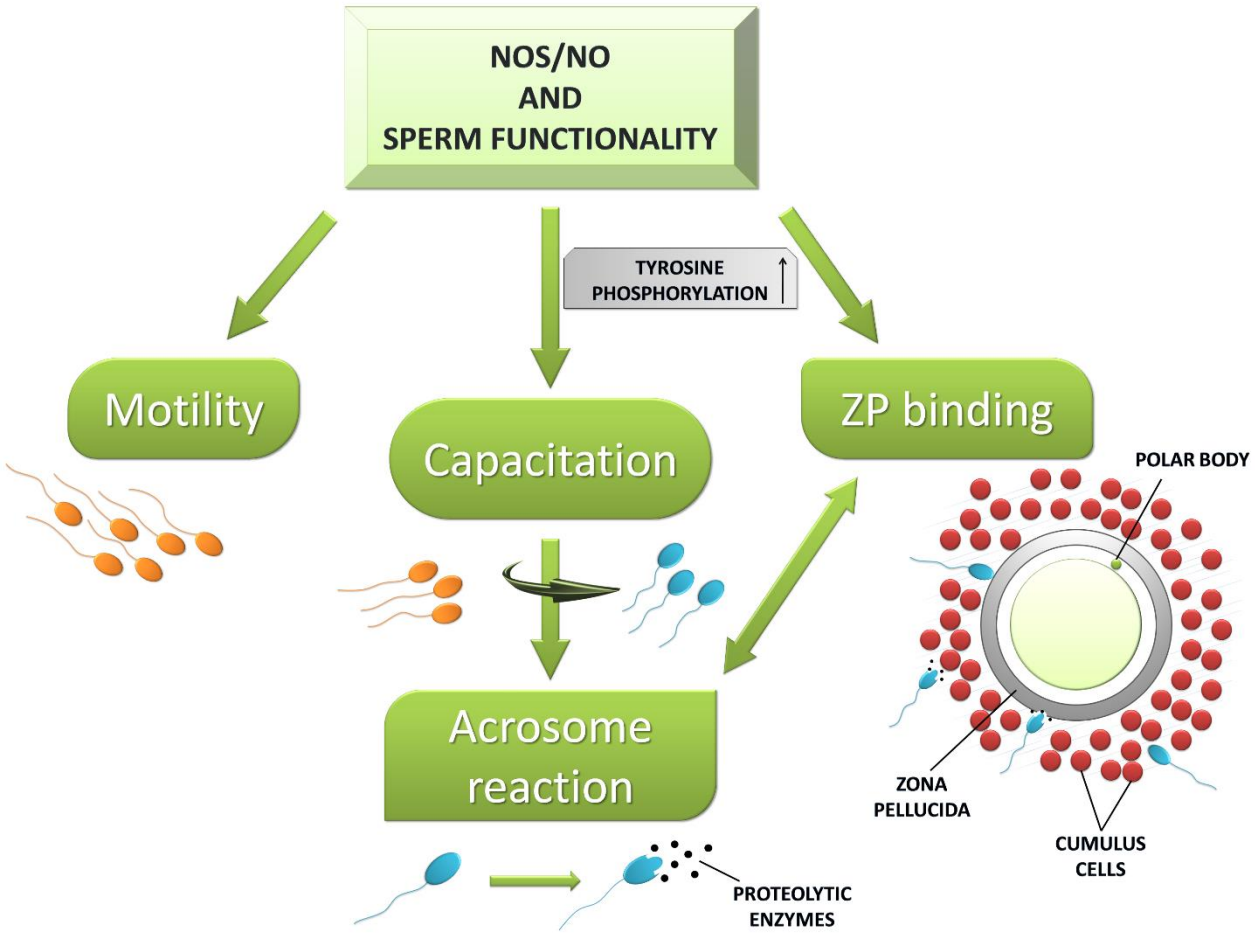


Figure 1. Some aspects of the sperm physiology modulated by the NOS/NO system. *At physiological levels, endogenous NO has a beneficial role in maintaining sperm motility, enhances tyrosine phosphorylation, which, in turn, promotes the capacitation process. NO also increases the sperm-zona pellucida binding ability and leads to a rise in the percentage of reacted spermatozoa, especially in the presence of follicular fluid or protein-enriched extracts of follicular fluid.*

It has been suggested that, upon approaching and entering the cumulus oophorus, both NO and progesterone, which are synthesized by the cumulus cells [47–51], provide a synergistic stimulus to

mobilize stored Ca^{2+} in the sperm neck/midpiece [52]. As a consequence, they can modulate flagellar activity and contribute to the hyperactivation that is vital for penetration of the oocyte vestments [53].

Interestingly, it has also been suggested that NO may exert a chemoattractant effect on spermatozoa. In fact, the percentage of mouse sperm migrating towards the medium containing a NO donor increased significantly [34]. Similar results were obtained when human spermatozoa were exposed to a NO donor [33]. In the latter case, the signal transduction pathway was also studied. It was proposed that NO exerts its chemoattractant effect through the activation of the NO/sGC/cGMP pathway, since the use of a NO scavenger and/or an sGC and cGMP-dependent protein kinase inhibitor reverted the NO donor-induced migration of sperm.

Since tyrosine phosphorylation in different sperm proteins is associated with the capacitation process [54], this aspect was investigated in order to further define the involvement of NO in capacitation. Herrero et al. [35] observed an increase in tyrosine phosphorylation when human sperm capacitation was accelerated by an NO-releasing compound. On the other hand, when sperm capacitation was inhibited by L-NAME, there was an attenuation in the tyrosine phosphorylation of sperm proteins. In addition, Thundathil et al. [36] reported that L-NAME prevented, and a NO donor promoted, the increase in threonine, glutamine and tyrosine phosphorylation in human spermatozoa. Furthermore, the addition of L-Arginine reversed the inhibitory effect of L-NAME on the capacitation and the associated increase in phosphorylation.

The correlation between NO and sperm-zona pellucida binding ability was investigated by Sengoku et al. [37], who reported that when treated with low concentrations of a NO donor, the number of spermatozoa which bind to the hemizona is higher than in sperm treated with a higher concentration. Additionally, a NO quencher lowered the enhancement of sperm binding by the NO donor.

NO also seems to modulate the acrosome reaction. The percentage of acrosome loss induced by human follicular fluid or by Ca^{2+} ionophore was studied when human spermatozoa were capacitated in the presence/absence of NO-releasing compounds or NOS inhibitors [35]. NO donors induced sperm cells to respond faster to human follicular fluid, whereas NOS inhibitors decreased the percentage of acrosome reaction. Similar results were obtained by Revelli et al. [38], who showed that different NO-releasing compounds were able to increase the percentage of reacted spermatozoa in the presence of protein-enriched extracts of human follicular fluid. Also, hemoglobin, a NO scavenger, inhibited the follicular fluid-induced acrosome reaction. In an in-depth analysis of the signaling pathway of the nitric oxide-induced acrosome reaction in human spermatozoa [39], the authors suggested that the acrosome reaction-inducing effect of exogenous NO on capacitated human

spermatozoa is accomplished via the NO/sGC/cGMP pathway, which leads to the activation of PKG. In fact, both the intracellular cGMP levels and the percentage of reacted spermatozoa were significantly increased after incubation with SNP. Furthermore, the SNP-induced acrosome reaction was significantly reduced in the presence of sGC inhibitors, a reduction that was reversed by the addition of a cell-permeating cGMP analogue to the incubation medium. Finally, PKG inhibition reduced the SNP-induced acrosome reaction.

2.3. NOS-activating molecules

As previously stated, NOS activity depends on the availability of its substrate and co-factors [10]. However, the scientific literature does not include many studies on the molecules present in the female reproductive tract which may activate, in one way or another, NOS enzymes in spermatozoa.

Starting from FF samples, Revelli et al. [38] obtained a protein-enriched follicular fluid solution (PFF), which was then used to study its effects on NOS activity, citrulline synthesis and acrosome reaction in human sperm. Interestingly, this study showed for the first time that the endogenous NOS activity of human sperm may be increased by PFF. Moreover, the authors demonstrated that PFF-mediated induction of sperm NOS activity leads to acrosome reaction in the same cells, thereby, establishing a link between follicle-derived substances, the activation of NO synthesis in sperm and biological responses.

Furthermore, the increase in NO synthesis mediated by PFF was not associated with a rise in the expression of NOS catalytic units, which is not surprising since specialized cells possess very poor, if any, transcriptional activity [38]. The authors hypothesized that PFF first determines the transient enzyme activation of sperm NOS, which is subsequently strengthened by a more stable modification of the enzyme.

However, more studies should be performed in order to identify the NOS-activating molecule(s) in the follicular fluid.

2.4. NO-mediated pathways in spermatozoa

In spermatozoa, NO acts via three main pathways (Fig. 2) [55]. Firstly, NO is able to activate sGC, leading to a rise in the intracellular levels of cGMP [56]. The latter activates the Cyclic Nucleotide-Gated Channels (CNG) localized in the flagellum of mammalian spermatozoa [57,58]. These channels seem to play an important role in the sperm motility control, by allowing the entry of Ca^{2+} ions to the cytoplasm during the capacitation process of mammal sperm [57]. Their activation is one of the first events that occurs during capacitation in the mouse spermatozoa [59]. cGMP also

activates PKG [60,61], which is involved in the serine/threonine phosphorylation of proteins that promote sperm capacitation and the acrosome reaction [62,63]. Furthermore, since cGMP and cAMP compete for the catalytic sites of phosphodiesterases [64,65], an increase in the intracytoplasmic cGMP concentration may inhibit cAMP degradation via cyclic nucleotide phosphodiesterase type 3 [66], thus increasing cAMP intracellular levels and activating PKA. The latter leads to an increase in protein tyrosine phosphorylation [67].

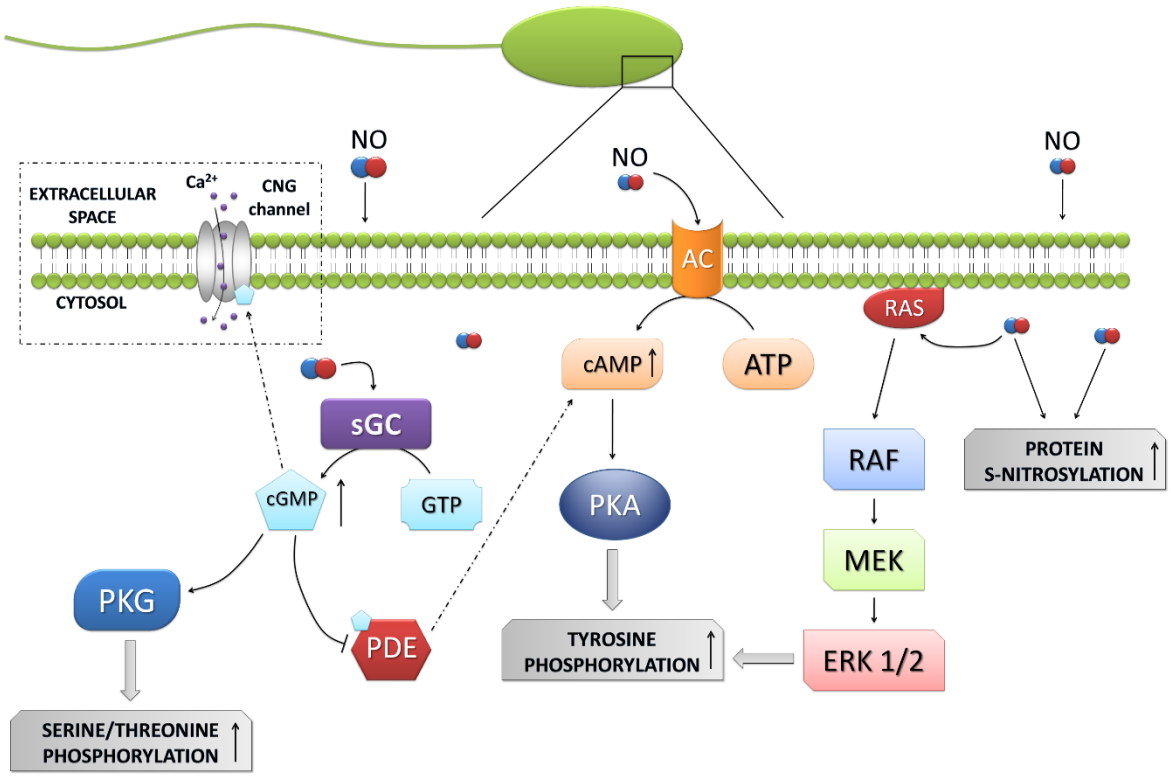


Figure 2. Representation of the main pathways through which NO acts in spermatozoa. *NO leads to an increase in the intracellular levels of cyclic guanosine monophosphate (cGMP) by activating the soluble isoform of Guanylate Cyclase (sGC). The cGMP can activate the Cyclic Nucleotide-Gated Channels (CNG) localized in the flagellum of mammalian spermatozoa, which regulate the influx of Ca^{2+} ions to the cytoplasm during the capacitation process, and also activates the cGMP-dependent Protein Kinase (PKG), leading to the serine/threonine phosphorylation of different proteins. It can also inhibit cyclic adenosine monophosphate (cAMP) degradation via cyclic nucleotide phosphodiesterase (PDE), which, leads to the activation of cAMP-dependent Protein Kinase (PKA) and tyrosine phosphorylation. Furthermore, NO is involved in the tyrosine phosphorylation process in a direct manner, by activating Adenylate Cyclase (AC) and the Extracellular Signal Regulated Kinase (ERK) pathway. Finally, NO determines post-translational protein modification in spermatozoa via S-nitrosylation.*

Secondly, NO is directly involved in tyrosine phosphorylation by modulating the cAMP/PKA and the ERK pathways. The cAMP/PKA pathway can be influenced by NO via activation of sGC (as described above), but it can also be regulated directly. In fact, S-nitrosylation of AC has been suggested as a possible mechanism of action of NO [68]. Low levels of NO may activate AC, consequently increasing the cAMP concentration and activating PKA [69]. However, high levels of NO can inhibit AC [68]. As far as the ERK pathway is concerned, NO reacts with the cysteine residues of the RAS protein, thus, activating it [70]. In turn, RAS triggers the RAF, MEK and ERK1/2 complex, necessary for tyrosine phosphorylation [71].

Thirdly, NO regulates the post-translational protein modification in spermatozoa via S-nitrosylation [11], a process similar to phosphorylation and acetylation [72,73]. S-nitrosylation consists of the covalent incorporation of NO into thiol groups (-SH) to form S-nitrosothiols (S-NO), a modification that is selective and reversible [55].

2.5. Function of S-nitrosoproteins in spermatozoa

An extensive study by Lefièvre et al. [11] described a large number of proteins present in the sperm of normozoospermic men, which can be subjected to S-nitrosylation in the presence of NO donors. Although the function of some nitrosylated proteins remains to be discovered, a considerable group of them are known to be metabolic proteins and proteins associated with energy generation and cell movement, suggesting a role for S-nitrosylation in sperm motility. This agrees with a previous proteomic analysis [74], in which the most abundant group was also involved in energy production.

Another considerable group of proteins were those involved in signal transduction, which agrees with a role for S-nitrosylation in modulating the sperm function [11]. Interestingly, since sperm are generally assumed to be transcriptionally inactive, a small percentage of the S-nitrosylated proteins identified by Lefièvre et al. [11] were related to transcription. Previous proteomic studies in sperm also observed the presence of proteins involved in transcription [74,75]. However, when comparing the human sperm S-nitrosoproteome with proteins identified during a proteomic study of sperm-oocyte interaction, only three proteins were found in common, suggesting that S-nitrosylation is not a regulatory mechanism employed during fertilization [11,76].

It is known that the mobilization of Ca^{2+} stored in the sperm neck/midpiece is necessary for the hyperactivation process [52]. The Ca^{2+} store in the neck of the sperm coincides with the region occupied by the Redundant Nuclear Envelope (RNE) [77] and, in order to mobilize Ca^{2+} from this site, Ryanodine Receptors (RyRs), which are intracellular Ca^{2+}-release channels involved in

regulation of cytosolic Ca^{2+} levels [78], need to be activated. These proteins contain a large number of thiol groups and are thus prone to S-nitrosylation by NO [11,79,80]. S-nitrosylation can potentiate the opening of RyRs [81–86], probably through the generation of the membrane permeant product S-nitrosocysteine [87]. It has been shown that an increase in Ca^{2+} induced by NO is accompanied by an increase in S-nitrosylation levels of endogenous RyRs [88,89], while these Ca^{2+} channels may be inhibited under strongly nitrosylating conditions or at high doses of NO (Fig. 3) [83,86,89]. Furthermore, progesterone acts synergistically with NO to mobilize Ca^{2+} in the sperm neck/midpiece by activation of RyRs [53], contributing to the hyperactivation process [55].

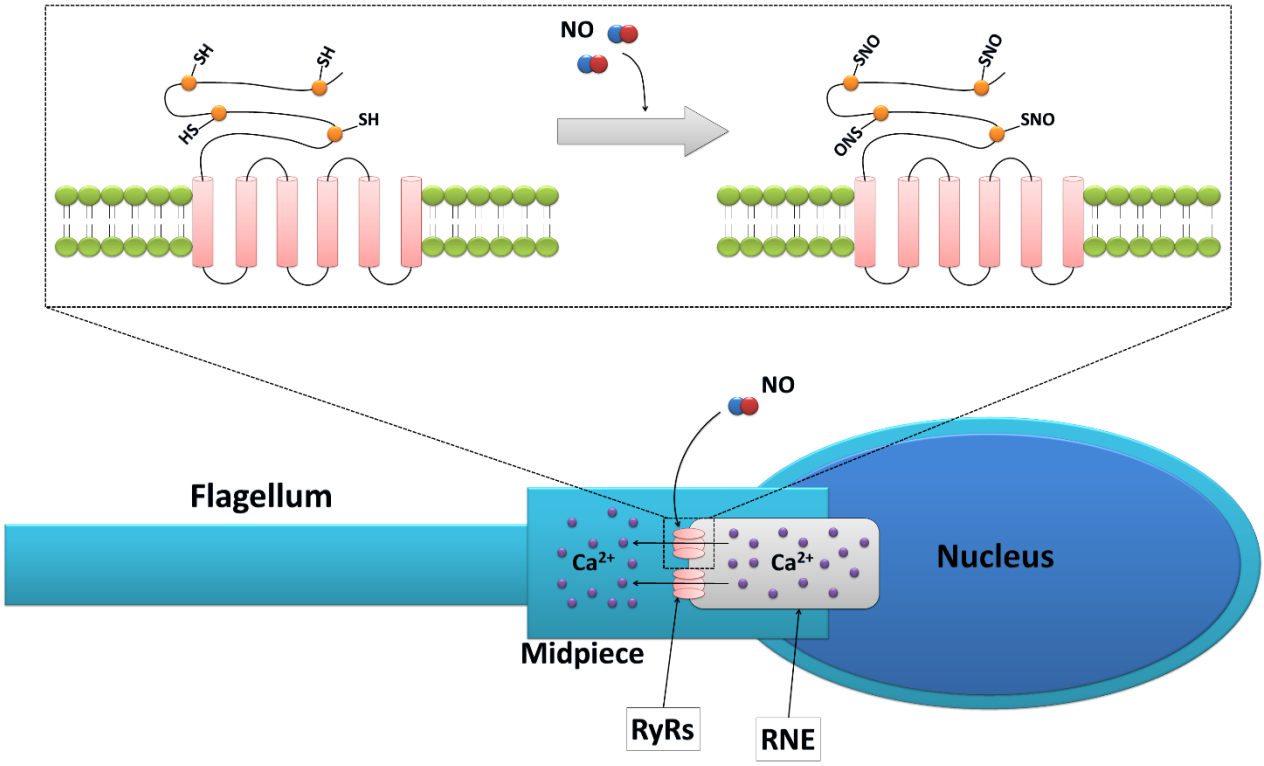


Figure 3. S-nitrosylation process. *NO acts on the thiol groups (-SH) of the cysteines in proteins to form S-nitrosothiols (S-NO). At the sperm neck/midpiece the S-nitrosylation occurs in Ryanodine Receptors (RyRs) allowing the release of calcium (Ca^{2+}) from the Redundant Nuclear Envelope (RNE), which is required for sperm hyperactivation. Adapted and modified from López-Úbeda and Matás (2015) [55].*

Other examples of proteins which can undergo S-nitrosylation in sperm and have a known biological significance are the A-Kinase anchoring proteins (AKAPs) [11]. Both AKAP3 and AKAP4 are present in the fibrous sheath of the sperm flagellum, control PKA activity and undergo phosphorylation during the capacitation process [90–92]. AKAP complexes also modulate the

motility of sperm. In fact, phosphodiesterase inhibitors were seen to significantly increase sperm motility [93], whereas PKA-anchoring inhibitor peptides arrested sperm motility [94].

A number of heat shock proteins (HSPs) may also be targets of S-nitrosylation in sperm [11] and some of them have been reported to act as important modulators of sperm capacitation. For instance, Asquith et al. [95] reported that heat shock protein 1 and endoplasmin undergo tyrosine phosphorylation during mouse sperm capacitation, whereas Nixon et al. [96] suggested that they form part of a zona pellucida complex, allowing successful sperm-egg interaction in the same species. Heat shock 70kDa protein 8 and heat shock protein 90α also undergo tyrosine phosphorylation during human sperm capacitation [90], but whether they function in a zona receptor complex is still unknown [11]. Furthermore, HspA2 has been shown to be a marker of sperm maturity [97], its expression in infertile men with idiopathic oligoteratozoospermia being lower than in normozoospermic men [98].

3. NOS/NO DUO IN OOCYTES

3.1 NOS expression

The synthesis of NO has been reported in oocytes, cumulus, corona cells [47,48] and in the oviduct [99,100] of several species [99,101,102]. In detail, Reyes et al. [47] used a cell-permeable fluorescent detector of NO synthesis on bovine oocytes and reported the presence of a uniform staining in the ovum cytoplasm. The fluorescent signal was also detected in the granulosa cells that were still attached to the ZP, but no staining was observed in the ZP.

The expression of NOS isoforms differs during the oestrous cycle in the follicles as well as in the oviduct [55]. Tao et al. [48] showed that in the porcine species the immunoreactivity of eNOS in early antral follicles was restricted to the oocyte and increased from small, medium to large follicle-enclosed oocytes. A weak eNOS immunoreactivity was observed in the cumulus cells from large follicles, but not in those from small and medium follicles. Furthermore, the authors reported the absence of immunoreactivity for iNOS in primordial, early antral follicle or the cumulus-oocyte complexes aspirated from small and medium follicles.

Nishikimi *et al*. [103] documented the presence of both eNOS and iNOS in the cytoplasm of murine oocytes. Moreover, an increase in NOS activity was observed in fertilized versus non-fertilized oocytes. Another study, in the same species, reported both these enzymes in oocytes, granulosa and theca cells, although iNOS mRNA was mostly distributed in granulosa cells and oocyte [104].

Previous studies evidenced the presence of NOS also in rat oocytes. Jablonka-Shariff *et al*. [105] reported a positive staining for eNOS in the cytoplasm of both ovarian and ovulated oocytes. Additionally, NOS mRNA were assayed in FF, granulosa and theca cells from preovulatory follicles in this species [106]. In the latter study, the authors observed an eNOS mRNA expression primarily in the theca cells, which increased after inducing the ovulation, but remained low in granulosa cells. On the other hand, iNOS mRNA expression was higher in granulosa cells and decreased significantly after the ovulation was induced.

As previously mentioned, the expression of NOS was reported in the oviduct as well. Cytosolic extracts from human ampullary segments and bovine ampullary segments can convert L-Arginine to L-Citrulline and release NO_2 and NO_3, respectively [99]. Furthermore, after culturing epithelial cells from these segments and subjecting them to an immunohistochemistry protocol, the presence of eNOS was demonstrated. In another work by Lapointe *et al*. [100] quantitative RT-PCR and immunohistochemical analyses were used to described the presence of all three NOS isoforms in bovine oviducts, where their activity has been shown to be regulated by hormones during the oestrous cycle. Similar results were reported in the epithelium of rat fallopian tubes [102].

When examining the ciliated epithelia in rat oviducts, the presence of eNOS was confirmed via WB and immunohistochemical staining [107]. It has been shown that oviductal epithelial cells produce endothelin, which is involved in the oviduct contraction [108,109]. Interestingly, after co-incubating bovine ampullary-isthmus segments with endothelin and a NOS inhibitor, the contracting effect of endothelin was significantly increased, thus suggesting a role for endogenous release of NO in the oviduct in its contraction [109]. Taken together, these data clearly suggest a role for NO in the transport of gametes and embryos during their transit in the oviduct.

3.2. NO and oocyte maturation

A considerable number of studies analyzed the effects of NO on the maturation process of the oocyte. In a publication by Jablonka-Shariff *et al*. [110] cumulus-oocyte complexes (COCs) were isolated from murine ovarian follicles, belonging to either wildtype or eNOS-knockout (eNOS-KO) females, and matured *in vitro* in the presence or not of a NOS inhibitor. The authors reported that a significantly smaller number of COCs were collected from eNOS-KO mice. Furthermore, in the presence of the NOS inhibitor, a lower percentage of oocytes reached maturity in both groups and, among them, several oocytes had an abnormal distribution of maternal chromosomes.

Mouse ovarian follicles were also cultured *in vitro* to test the effect of adding or not L-Arginine in the medium [104]. Interestingly, the absence of L-Arginine led to a significant decrease in the follicle survival and ovulation rates.

On the other hand, Nakamura *et al*. [111] reported that a NO donor inhibited oocyte maturation in rats, whereas a selective iNOS inhibitor promoted it. The same authors localized the iNOS isoform mainly in the granulosa cells and its expression was described as lower after inducing the ovulation [106]. Since progesterone synthesis is inhibited in the presence of high levels of NO [112] and the latter cause apoptosis and increased DNA fragmentation in rat granulosa cells [106], the authors suggested that the modulation of iNOS-derived NO is particularly important during maturation in rats.

The importance of NO during the maturation process was also investigated in porcine oocytes, in which the meiotic resumption and the extrusion of the first polar body were reported to be significantly decreased by NOS inhibitors [48]. Additionally, when analyzing the cumulus expansion during *in vitro* maturation (IVM), Romero-Aguirregomezcorta *et al*. [113] observed that three different NOS inhibitors diminished significantly this parameter, whereas no effect was reported when the IVM medium was supplemented with a NO donor. Interestingly, in this study, the authors demonstrated the importance of protein S-nitrosylation during porcine IVM. Particularly, when iNOS was inhibited the amount of S-nitrosylation decreased, as well as the meiotic resumption.

Another study reported that the exposure of mouse oocytes to a NO donor led to a significant reduction in the ZP dissolution time and spontaneous cortical granule exocytosis [114]. Moreover, the exogenous NO also decreased the rate of spindle abnormalities, therefore, suggesting a role for NO in delaying the oocyte aging [114].

Even though the way NO acts during the meiotic resumption remains opened for discussion, it has been proposed that low intraoocyte concentrations of NO may facilitate this process possibly through the cGMP-mediated pathway, known to be upregulated by NO [115]. Particularly, reduced NO levels can be translated to decreased cGMP levels, which in turn activates the cAMP-phosphodiesterase type 3. The latter reduces the oocyte's inner levels of cAMP, consequently, inactivating PKA and triggering a cascade of events which end with the destabilization of the Maturation Promoting Factor and trigger meiotic resumption (summarized by [115]).

3.3. Role of NO in embryos

Not surprisingly, the synthesis and importance of NO in embryos was also studied. A decrease in NOS activity was described by Nishikimi *et al*. [103] in mice embryos between the two-cell and the eight-cell stage, but it increased at the morula stage, specifically in the inner cell population. Moreover, expanded blastocysts showed NOS activity only in the inner cell mass (ICM). The authors also described the localization of eNOS and iNOS via immunocytochemical staining. Both eNOS and iNOS were identified in the cytoplasm of embryos up to the blastocyst stage. Particularly, eNOS was also distributed in the nuclei of embryos, but with a lower intensity starting from the two-cell stage. As far as iNOS is concerned, the morula outer cell population and the trophectoderm cells in blastocyst presented a more intense staining than the respective inner cells.

In a pilot study, the secretion of NO was assessed in embryos, which were later transferred to patients undergoing infertility treatment [116]. The authors did not observe significant differences in the levels of NO_2 and NO_3 measured in the culture medium between the grade A, grade B or grade C+D embryos. Interestingly though, when comparing the patients who did not obtain a pregnancy with those who did, the NO_2/NO_3 concentrations were significantly higher in the latter group.

Barroso *et al*. [117] analyzed in mice how NO affected embryo development *in vitro* and the implantation of embryos *in vivo*. Two-cell embryos were incubated with increased concentrations of a NO donor, whereas *in vivo* the same donor was administered subcutaneously. The authors reported a cytotoxic effect of high concentrations of NO, which caused an inhibition of both embryo development *in vitro* and *in vivo* implantation.

Clinical studies reported interesting results when investigating the association between the NO concentration inside the follicle and embryo cleavage. For instance, Barrionuevo *et al*. [15] observed that follicles which contained oocytes capable of dividing beyond the 6-cell stage, once fertilized, possessed lower FF levels of NO_3/NO_2. Consistent with these findings, a higher NO concentration in the FF was correlated with severe embryo fragmentation and asymmetry. In contrast, Manau *et al*. [118] did not identify any correlation between FF NO levels and embryo grading.

NOS have also been described in the endometrium, where NO may have important functions particularly during embryo implantation and pregnancy [119]. The synthesis of L-citrulline from L-Arginine and, consequently, NO_2 and NO_3, was reported in uterine tissues from pregnant rats and it was suggested that NO could contribute to the myometrial relaxation during pregnancy [120]. Furthermore, eNOS immunoreactivity was localized in the human placenta [121–123], where NO

may regulate the placental blood flow and limit platelet aggregation at the interface between maternal and fetal circulations [124].

4. CONCLUDING REMARKS

In recent years, our knowledge of the involvement of the NOS/NO system in mammalian fertilization has grown, and there is clear evidence that NO acts as a significant modulator of the male and female gamete. However, some aspects regarding the NOS/NO duo, such as how the biological function of the S-nitrosylated proteins changes or which are the targets of NO-mediated phosphorylation and what they, in turn, modulate, need to be better understood in most species. Shedding light on these mechanisms will help discover more on the etiopathology of subfertility/infertility problems and how such problems can be overcome.

These considerations led us to decide to further investigate in the present thesis the role of NO in relation to boar and human sperm capacitation. At first, we analyzed how the addition of NO or its inhibition affected sperm capacitation and IVF in the porcine species. Next, we determined how protein phosphorylation is modulated by NO, L-Arginine and FF during human sperm capacitation and, finally, we examined the repercussion of FF NO levels on the quality of oocytes in stimulated cycles.

5. REFERENCES

[1] Sharlip ID, Jarow JP, Belker AM, Lipshultz LI, Sigman M, Thomas AJ, et al. Best practice policies for male infertility. Fertil Steril 2002;77:873–82.

[2] Buzadzic B, Vucetic M, Jankovic A, Stancic A, Korac A, Korac B, et al. New insights into male (in)fertility: the importance of NO. Br J Pharmacol 2015;172:1455–67. doi:10.1111/bph.12675.

[3] Agarwal A, Allamaneni SSR. Role of free radicals in female reproductive diseases and assisted reproduction. Reprod Biomed Online 2004;9:338–47.

[4] Dawson EB, Harris WA, Teter MC, Powell LC. Effect of ascorbic acid supplementation on the sperm quality of smokers. Fertil Steril 1992;58:1034–9.

[5] Kessopoulou E, Powers HJ, Sharma KK, Pearson MJ, Russell JM, Cooke ID, et al. A double-blind randomized placebo cross-over controlled trial using the antioxidant vitamin E to treat reactive oxygen species associated male infertility. Fertil Steril 1995;64:825–31.

[6] Pacher P, Beckman JS, Liaudet L. Nitric Oxide and Peroxynitrite in Health and Disease. Physiol Rev 2007;87:315–424. doi:10.1152/physrev.00029.2006.

[7] Förstermann U, Sessa WC. Nitric oxide synthases: regulation and function. Eur Heart J 2012;33:829–37, 837a-837d. doi:10.1093/eurheartj/ehr304.

[8] Griffith OW, Stuehr DJ. Nitric oxide synthases: properties and catalytic mechanism. Annu Rev Physiol 1995;57:707–36. doi:10.1146/annurev.ph.57.030195.003423.

[9] Snyder SH. Nitric oxide. No endothelial NO. Nature 1995;377:196–7. doi:10.1038/377196a0.

[10] Rosselli M, Keller PJ, Dubey RK. Role of nitric oxide in the biology, physiology and pathophysiology of reproduction. Hum Reprod Update 1998;4:3–24.

[11] Lefièvre L, Chen Y, Conner SJ, Scott JL, Publicover SJ, Ford WCL, et al. Human spermatozoa contain multiple targets for protein S-nitrosylation: An alternative mechanism of the modulation of sperm function by nitric oxide? Proteomics 2007;7:3066–84. doi:10.1002/pmic.200700254.

[12] Herrero MB, Viggiano JM, Pérez Martínez S, de Gimeno MF. Evidence that nitric oxide synthase is involved in progesterone-induced acrosomal exocytosis in mouse spermatozoa. Reprod Fertil Dev 1997;9:433–9.

[13] Gawronska B, Bodek G, Ziecik AJ. Distribution of NADPH-diaphorase and Nitric Oxide Synthase (NOS) in Different Regions of Porcine Oviduct During the Estrous Cycle. J Histochem Cytochem 2000;48:867–75. doi:10.1177/002215540004800615.

[14] Revelli A, Delle Piane L, Casano S, Molinari E, Massobrio M, Rinaudo P. Follicular fluid content and oocyte quality: from single biochemical markers to metabolomics. Reprod Biol Endocrinol 2009;7:40. doi:10.1186/1477-7827-7-40.

[15] Barrionuevo MJ, Schwandt RA, Rao PS, Graham LB, Maisel LP, Yeko TR. Nitric oxide (NO) and interleukin-1beta (IL-1beta) in follicular fluid and their correlation with fertilization and embryo cleavage. Am J Reprod Immunol 2000;44:359–64.

[16] Barroso G, Barrionuevo M, Rao P, Graham L, Danforth D, Huey S, et al. Vascular endothelial growth factor, nitric oxide, and leptin follicular fluid levels correlate negatively with embryo quality in IVF patients. Fertil Steril 1999;72:1024–6.

[17] Goud PT, Goud AP, Joshi N, Puscheck E, Diamond MP, Abu-Soud HM. Dynamics of nitric oxide, altered follicular microenvironment, and oocyte quality in women with endometriosis. Fertil Steril 2014;102:151-159.e5. doi:10.1016/j.fertnstert.2014.03.053.

[18] Lee KS, Joo BS, Na YJ, Yoon MS, Choi OH, Kim WW. Relationships between concentrations of tumor necrosis factor-alpha and nitric oxide in follicular fluid and oocyte quality. J Assist Reprod Genet 2000;17:222–8.

[19] Vignini A, Turi A, Giannubilo SR, Pescosolido D, Scognamiglio P, Zanconi S, et al. Follicular fluid nitric oxide (NO) concentrations in stimulated cycles: the relationship to embryo grading. Arch Gynecol Obstet 2008;277:229–32. doi:10.1007/s00404-007-0445-y.

[20] Yalçınkaya E, Cakıroğlu Y, Doğer E, Budak O, Cekmen M, Calışkan E. Effect of follicular fluid NO, MDA and GSH levels on in vitro fertilization outcomes. J Turkish Ger Gynecol Assoc 2013;14:136–41. doi:10.5152/jtgga.2013.53323.

[21] Kim KH, Oh DS, Jeong JH, Shin BS, Joo BS, Lee KS. Follicular blood flow is a better predictor of the outcome of in vitro fertilization-embryo transfer than follicular fluid vascular endothelial growth factor and nitric oxide concentrations. Fertil Steril 2004;82:586–92. doi:10.1016/j.fertnstert.2004.02.120.

[22] Herrero MB, Pérez Martínez S, Viggiano JM, Polak JM, de Gimeno MF. Localization by indirect immunofluorescence of nitric oxide synthase in mouse and human spermatozoa. Reprod Fertil Dev 1996;8:931–4.

[23] O'Bryan MK, Zini A, Cheng CY, Schlegel PN. Human sperm endothelial nitric oxide synthase expression: correlation with sperm motility. Fertil Steril 1998;70:1143–7. doi:10.1016/S0015-0282(98)00382-3.

[24] Herrero MB, Goin JC, Boquet M, Canteros MG, Franchi AM, Perez Martinez S, et al. The nitric oxide synthase of mouse spermatozoa. FEBS Lett 1997;411:39–42.

[25] Meiser H, Schulz R. Detection and localization of two constitutive NOS isoforms in bull spermatozoa. Anat Histol Embryol 2003;32:321–5.

[26] Hou M-L, Huang S-Y, Lai Y-K, Lee W-C. Geldanamycin augments nitric oxide production and promotes capacitation in boar spermatozoa. Anim Reprod Sci 2008;104:56–68. doi:10.1016/j.anireprosci.2007.01.006.

[27] Aquila S, Giordano F, Guido C, Rago V, Carpino A. Nitric oxide involvement in the acrosome reaction triggered by leptin in pig sperm. Reprod Biol Endocrinol 2011;9:133. doi:10.1186/1477-7827-9-133.

[28] Ortega Ferrusola C, González Fernández L, Macías García B, Salazar-Sandoval C, Morillo Rodríguez A, Rodríguez Martinez H, et al. Effect of cryopreservation on nitric oxide production by stallion spermatozoa. Biol Reprod 2009;81:1106–11. doi:10.1095/biolreprod.109.078220.

[29] Liman N, Alan E. Region-specific localization of NOS isoforms and NADPH-diaphorase activity in the intratesticular and excurrent duct systems of adult domestic cats (Felis catus). Microsc Res Tech 2016;79:192–208. doi:10.1002/jemt.22619.

[30] Guevara I, Iwanejko J, Dembińska-Kieć A, Pankiewicz J, Wanat A, Anna P, et al. Determination of nitrite/nitrate in human biological material by the simple Griess reaction. Clin Chim Acta 1998;274:177–88.

[31] Lewis SE, Donnelly ET, Sterling ES, Kennedy MS, Thompson W, Chakravarthy U. Nitric oxide synthase and nitrite production in human spermatozoa: evidence that endogenous nitric oxide is beneficial to sperm motility. Mol Hum Reprod 1996;2:873–8.

[32] Donnelly ET, Lewis SE, Thompson W, Chakravarthy U. Sperm nitric oxide and motility: the effects of nitric oxide synthase stimulation and inhibition. Mol Hum Reprod 1997;3:755–62.

[33] Miraglia E, Rullo ML, Bosia A, Massobrio M, Revelli A, Ghigo D. Stimulation of the nitric oxide/cyclic guanosine monophosphate signaling pathway elicits human sperm chemotaxis in vitro. Fertil Steril 2007;87:1059–63. doi:10.1016/j.fertnstert.2006.07.1540.

[34] Sliwa L, Stochmal E. Effect of sodium nitroprusside on mouse sperm migration in vitro. Arch Androl 2000;45:29–33.

[35] Herrero MB, de Lamirande E, Gagnon C. Nitric oxide regulates human sperm capacitation and protein-tyrosine phosphorylation in vitro. Biol Reprod 1999;61:575–81. doi:10.1095/biolreprod61.3.575.

[36] Thundathil J, de Lamirande E, Gagnon C. Nitric oxide regulates the phosphorylation of the threonine-glutamine-tyrosine motif in proteins of human spermatozoa during capacitation. Biol Reprod 2003;68:1291–8. doi:10.1095/biolreprod.102.008276.

[37] Sengoku K, Tamate K, Yoshida T, Takaoka Y, Miyamoto T, Ishikawa M. Effects of low concentrations of nitric oxide on the zona pellucida binding ability of human spermatozoa. Fertil Steril 1998;69:522–7.

[38] Revelli A, Soldati G, Costamagna C, Pellerey O, Aldieri E, Massobrio M, et al. Follicular fluid proteins stimulate nitric oxide (NO) synthesis in human sperm: a possible role for NO in acrosomal reaction. J Cell Physiol 1999;178:85–92. doi:10.1002/(SICI)1097-4652(199901)178:1<85::AID-JCP11>3.0.CO;2-Y.

[39] Revelli A, Costamagna C, Moffa F, Aldieri E, Ochetti S, Bosia A, et al. Signaling pathway of nitric oxide-induced acrosome reaction in human spermatozoa. Biol Reprod 2001;64:1708–12.

[40] Herrero MB, Cebral E, Boquet M, Viggiano JM, Vitullo A, Gimeno MA. Effect of nitric oxide on mouse sperm hyperactivation. Acta Physiol Pharmacol Ther Latinoam 1994;44:65–9.

[41] Creech MM, Arnold E V, Boyle B, Muzinich MC, Montville C, Bohle DS, et al. Sperm motility enhancement by nitric oxide produced by the oocytes of fathead minnows, Pimephelas promelas. J Androl 1998;19:667–74.

[42] Yeoman RR, Jones WD, Rizk BM. Evidence for nitric oxide regulation of hamster sperm hyperactivation. J Androl 1998;19:58–64.

[43] Hellstrom WJ, Bell M, Wang R, Sikka SC. Effect of sodium nitroprusside on sperm motility, viability, and lipid peroxidation. Fertil Steril 1994;61:1117–22.

[44] Rosselli M, Dubey RK, Imthurn B, Macas E, Keller PJ. Effects of nitric oxide on human spermatozoa: evidence that nitric oxide decreases sperm motility and induces sperm toxicity. Hum Reprod 1995;10:1786–90.

[45] Weinberg JB, Doty E, Bonaventura J, Haney AF. Nitric oxide inhibition of human sperm motility. Fertil Steril 1995;64:408–13.

[46] Tomlinson MJ, East SJ, Barratt CL, Bolton AE, Cooke ID. Preliminary communication: possible role of reactive nitrogen intermediates in leucocyte-mediated sperm dysfunction. Am J Reprod Immunol 1992;27:89–92.

[47] Reyes R, Vázquez MLS, Delgado NM. Detection and bioimaging of nitric oxide in bovine oocytes and sperm cells. Arch Androl 2004;50:303–9. doi:10.1080/01485010490448471.

[48] Tao Y, Fu Z, Zhang M, Xia G, Yang J, Xie H. Immunohistochemical localization of inducible and endothelial nitric oxide synthase in porcine ovaries and effects of NO on antrum formation and oocyte meiotic maturation. Mol Cell Endocrinol 2004;222:93–103. doi:10.1016/j.mce.2004.04.014.

[49] Chian RC, Ao A, Clarke HJ, Tulandi T, Tan SL. Production of steroids from human cumulus cells treated with different concentrations of gonadotropins during culture in vitro. Fertil Steril 1999;71:61–6.

[50] Mingoti GZ, Garcia JM, Rosa-e-Silva AAM. Steroidogenesis in cumulus cells of bovine cumulus-oocyte-complexes matured in vitro with BSA and different concentrations of steroids. Anim Reprod Sci 2002;69:175–86.

[51] Yamashita Y, Shimada M, Okazaki T, Maeda T, Terada T. Production of progesterone from de novo-synthesized cholesterol in cumulus cells and its physiological role during meiotic resumption of porcine oocytes. Biol Reprod 2003;68:1193–8. doi:10.1095/biolreprod.102.010934.

[52] Bedu-Addo K, Costello S, Harper C, Machado-Oliveira G, Lefievre L, Ford C, et al. Mobilisation of stored calcium in the neck region of human sperm--a mechanism for regulation of flagellar activity. Int J Dev Biol 2008;52:615–26. doi:10.1387/ijdb.072535kb.

[53] Machado-Oliveira G, Lefievre L, Ford C, Herrero MB, Barratt C, Connolly TJ, et al. Mobilisation of Ca2+ stores and flagellar regulation in human sperm by S-nitrosylation: a role for NO synthesised in the female reproductive tract. Development 2008;135:3677–86. doi:10.1242/dev.024521.

[54] Emiliozzi C, Fenichel P. Protein tyrosine phosphorylation is associated with capacitation of human sperm in vitro but is not sufficient for its completion. Biol Reprod 1997;56:674–9. doi:10.1095/biolreprod56.3.674.

[55] López-Úbeda R, Matás C. An Approach to the Factors Related to Sperm Capacitation Process. Andrology 2015;4. doi:10.4172/2167-0250.1000128.

[56] Murad F. The nitric oxide-cyclic GMP signal transduction system for intracellular and intercellular communication. Recent Prog Horm Res 1994;49:239–48.

[57] Wiesner B, Weiner J, Middendorff R, Hagen V, Kaupp UB, Weyand I. Cyclic nucleotide-gated channels on the flagellum control Ca2+ entry into sperm. J Cell Biol 1998;142:473–84.

[58] Weyand I, Godde M, Frings S, Weiner J, Müller F, Altenhofen W, et al. Cloning and functional expression of a cyclic-nucleotide-gated channel from mammalian sperm. Nature 1994;368:859–63. doi:10.1038/368859a0.

[59] Cisneros-Mejorado A, Hernández-Soberanis L, Islas-Carbajal MC, Sánchez D. Capacitation and Ca(2+) influx in spermatozoa: role of CNG channels and protein kinase G. Andrology 2014;2:145–54. doi:10.1111/j.2047-2927.2013.00169.x.

[60] Lohmann SM, Vaandrager AB, Smolenski A, Walter U, De Jonge HR. Distinct and specific functions of cGMP-dependent protein kinases. Trends Biochem Sci 1997;22:307–12.

[61] Pfeifer A, Ruth P, Dostmann W, Sausbier M, Klatt P, Hofmann F. Structure and function of cGMP-dependent protein kinases. Rev Physiol Biochem Pharmacol 1999;135:105–49.

[62] Rahman MS, Kwon W-S, Pang M-G. Calcium influx and male fertility in the context of the sperm proteome: an update. Biomed Res Int 2014;2014:841615. doi:10.1155/2014/841615.

[63] Miraglia E, De Angelis F, Gazzano E, Hassanpour H, Bertagna A, Aldieri E, et al. Nitric oxide stimulates human sperm motility via activation of the cyclic GMP/protein kinase G signaling pathway. Reproduction 2011;141:47–54. doi:10.1530/REP-10-0151.

[64] Bender AT, Beavo JA. Cyclic Nucleotide Phosphodiesterases: Molecular Regulation to Clinical Use. Pharmacol Rev 2006;58:488–520. doi:10.1124/pr.58.3.5.

[65] Conti M, Beavo J. Biochemistry and physiology of cyclic nucleotide phosphodiesterases: essential components in cyclic nucleotide signaling. Annu Rev Biochem 2007;76:481–511. doi:10.1146/annurev.biochem.76.060305.150444.

[66] Kurtz A, Götz KH, Hamann M, Wagner C. Stimulation of renin secretion by nitric oxide is mediated by phosphodiesterase 3. Proc Natl Acad Sci U S A 1998;95:4743–7.

[67] Beavo JA. Cyclic nucleotide phosphodiesterases: functional implications of multiple isoforms. Physiol Rev 1995;75:725–48. doi:10.1152/physrev.1995.75.4.725.

[68] McVey M, Hill J, Howlett A, Klein C. Adenylyl cyclase, a coincidence detector for nitric oxide. J Biol Chem 1999;274:18887–92.

[69] Belén Herrero M, Chatterjee S, Lefièvre L, De Lamirande E, Gagnon C. Nitric oxide interacts with the cAMP pathway to modulate capacitation of human spermatozoa. Free Radic Biol Med 2000;29:522–36. doi:10.1016/S0891-5849(00)00339-7.

[70] O'Flaherty C, de Lamirande E, Gagnon C. Positive role of reactive oxygen species in mammalian sperm capacitation: triggering and modulation of phosphorylation events. Free Radic Biol Med 2006;41:528–40. doi:10.1016/j.freeradbiomed.2006.04.027.

[71] de Lamirande E. The extracellular signal-regulated kinase (ERK) pathway is involved in human sperm function and modulated by the superoxide anion. Mol Hum Reprod 2002;8:124–35. doi:10.1093/molehr/8.2.124.

[72] Foster MW, Stamler JS. New insights into protein S-nitrosylation. Mitochondria as a model system. J Biol Chem 2004;279:25891–7. doi:10.1074/jbc.M313853200.

[73] Hess DT, Matsumoto A, Kim S-O, Marshall HE, Stamler JS. Protein S-nitrosylation: purview and parameters. Nat Rev Mol Cell Biol 2005;6:150–66. doi:10.1038/nrm1569.

[74] Martínez-Heredia J, Estanyol JM, Ballescà JL, Oliva R. Proteomic identification of human sperm proteins. Proteomics 2006;6:4356–69. doi:10.1002/pmic.200600094.

[75] Lalancette C, Faure RL, Leclerc P. Identification of the proteins present in the bull sperm cytosolic fraction enriched in tyrosine kinase activity: a proteomic approach. Proteomics 2006;6:4523–40. doi:10.1002/pmic.200500578.

[76] Stein KK, Go JC, Lane WS, Primakoff P, Myles DG. Proteomic analysis of sperm regions that mediate sperm-egg interactions. Proteomics 2006;6:3533–43. doi:10.1002/pmic.200500845.

[77] Costello S, Michelangeli F, Nash K, Lefievre L, Morris J, Machado-Oliveira G, et al. Ca2+-stores in sperm: their identities and functions. Reproduction 2009;138:425–37. doi:10.1530/REP-09-0134.

[78] Kakizawa S, Yamazawa T, Iino M. Nitric oxide-induced calcium release: activation of type 1 ryanodine receptor by endogenous nitric oxide. Channels (Austin) 2013;7:1–5. doi:10.4161/chan.22555.

[79] Takeshima H, Nishimura S, Matsumoto T, Ishida H, Kangawa K, Minamino N, et al. Primary structure and expression from complementary DNA of skeletal muscle ryanodine receptor. Nature 1989;339:439–45. doi:10.1038/339439a0.

[80] Otsu K, Willard HF, Khanna VK, Zorzato F, Green NM, MacLennan DH. Molecular cloning of cDNA encoding the Ca2+ release channel (ryanodine receptor) of rabbit cardiac muscle sarcoplasmic reticulum. J Biol Chem 1990;265:13472–83.

[81] Stoyanovsky D, Murphy T, Anno PR, Kim YM, Salama G. Nitric oxide activates skeletal and cardiac ryanodine receptors. Cell Calcium 1997;21:19–29.

[82] Xu L, Eu JP, Meissner G, Stamler JS. Activation of the cardiac calcium release channel (ryanodine receptor) by poly-S-nitrosylation. Science 1998;279:234–7.

[83] Hart JD, Dulhunty AF. Nitric oxide activates or inhibits skeletal muscle ryanodine receptors depending on its concentration, membrane potential and ligand binding. J Membr Biol 2000;173:227–36.

[84] Li N, Zou AP, Ge ZD, Campbell WB, Li PL. Effect of nitric oxide on calcium-induced calcium release in coronary arterial smooth muscle. Gen Pharmacol 2000;35:37–45.

[85] Heunks LM, Machiels HA, Dekhuijzen PN, Prakash YS, Sieck GC. Nitric oxide affects sarcoplasmic calcium release in skeletal myotubes. J Appl Physiol 2001;91:2117–24. doi:10.1152/jappl.2001.91.5.2117.

[86] Zima A, Blatter L. Redox regulation of cardiac calcium channels and transporters. Cardiovasc Res 2006;71:310–21. doi:10.1016/j.cardiores.2006.02.019.

[87] Zhang Y, Hogg N. The mechanism of transmembrane S-nitrosothiol transport. Proc Natl Acad Sci 2004;101:7891–6. doi:10.1073/pnas.0401167101.

[88] Kakizawa S. Nitric Oxide-Induced Calcium Release: Activation of Type 1 Ryanodine Receptor, a Calcium Release Channel, through Non-Enzymatic Post-Translational Modification by Nitric Oxide. Front Endocrinol (Lausanne) 2013;4:142. doi:10.3389/fendo.2013.00142.

[89] Zahradníková A, Minarovic I, Venema RC, Mészáros LG. Inactivation of the cardiac ryanodine receptor calcium release channel by nitric oxide. Cell Calcium 1997;22:447–54.

[90] Ficarro S, Chertihin O, Westbrook VA, White F, Jayes F, Kalab P, et al. Phosphoproteome analysis of capacitated human sperm. Evidence of tyrosine phosphorylation of a kinase-anchoring protein 3 and valosin-containing protein/p97 during capacitation. J Biol Chem 2003;278:11579–89. doi:10.1074/jbc.M202325200.

[91] Visconti PE, Johnson LR, Oyaski M, Fornés M, Moss SB, Gerton GL, et al. Regulation, localization, and anchoring of protein kinase A subunits during mouse sperm capacitation. Dev Biol 1997;192:351–63. doi:10.1006/dbio.1997.8768.

[92] Bajpai M, Fiedler SE, Huang Z, Vijayaraghavan S, Olson GE, Livera G, et al. AKAP3 Selectively Binds PDE4A Isoforms in Bovine Spermatozoa1. Biol Reprod 2006;74:109–18. doi:10.1095/biolreprod.105.043588.

[93] Fisch JD, Behr B, Conti M. Enhancement of motility and acrosome reaction in human spermatozoa: differential activation by type-specific phosphodiesterase inhibitors. Hum Reprod 1998;13:1248–54. doi:10.1093/humrep/13.5.1248.

[94] Vijayaraghavan S, Goueli SA, Davey MP, Carr DW. Protein kinase A-anchoring inhibitor peptides arrest mammalian sperm motility. J Biol Chem 1997;272:4747–52. doi:10.1074/jbc.272.8.4747.

[95] Asquith KL, Baleato RM, McLaughlin EA, Nixon B, Aitken RJ. Tyrosine phosphorylation activates surface chaperones facilitating sperm-zona recognition. J Cell Sci 2004;117:3645–57. doi:10.1242/jcs.01214.

[96] Nixon B, Asquith KL, John Aitken R. The role of molecular chaperones in mouse sperm–egg interactions. Mol Cell Endocrinol 2005;240:1–10. doi:10.1016/j.mce.2005.06.004.

[97] Ergur AR, Dokras A, Giraldo JL, Habana A, Kovanci E, Huszar G. Sperm maturity and treatment choice of in vitro fertilization (IVF) or intracytoplasmic sperm injection: diminished sperm HspA2 chaperone levels predict IVF failure. Fertil Steril 2002;77:910–8.

[98] Cedenho AP, Lima SB, Cenedeze MA, Spaine DM, Ortiz V, Oehninger S. Oligozoospermia and heat-shock protein expression in ejaculated spermatozoa. Hum Reprod 2006;21:1791–4. doi:10.1093/humrep/del055.

[99] Rosselli M, Dubey RK, Rosselli MA, Macas E, Fink D, Lauper U, et al. Identification of nitric oxide synthase in human and bovine oviduct. Mol Hum Reprod 1996;2:607–12.

[100] Lapointe J, Roy M, St-Pierre I, Kimmins S, Gauvreau D, MacLaren LA, et al. Hormonal and spatial regulation of nitric oxide synthases (NOS) (neuronal NOS, inducible NOS, and endothelial NOS) in the oviducts. Endocrinology 2006;147:5600–10. doi:10.1210/en.2005-1548.

[101] Ekerhovd E, Brännström M, Alexandersson M, Norström A. Evidence for nitric oxide mediation of contractile activity in isolated strips of the human Fallopian tube. Hum Reprod 1997;12:301–5. doi:10.1093/humrep/12.2.301.

[102] Bryant CE, Tomlinson A, Mitchell JA, Thiemermann C, Willoughby DA. Nitric oxide synthase in the rat fallopian tube is regulated during the oestrous cycle. J Endocrinol 1995;146:149–57.

[103] Nishikimi A, Matsukawa T, Hoshino K, Ikeda S, Kira Y, Sato EF, et al. Localization of nitric oxide synthase activity in unfertilized oocytes and fertilized embryos during preimplantation development in mice. Reproduction 2001;122:957–63.

[104] Mitchell LM, Kennedy CR, Hartshorne GM. Expression of nitric oxide synthase and effect of substrate manipulation of the nitric oxide pathway in mouse ovarian follicles. Hum Reprod 2004;19:30–40. doi:10.1093/humrep/deh032.

[105] Jablonka-Shariff A, Basuray R, Olson LM. Inhibitors of nitric oxide synthase influence oocyte maturation in rats. J Soc Gynecol Investig 1999;6:95–101.

[106] Yamagata Y, Nakamura Y, Sugino N, Harada A, Takayama H, Kashida S, et al. Alterations in nitrate/nitrite and nitric oxide synthase in preovulatory follicles in gonadotropin-primed immature rat. Endocr J 2002;49:219–26.

[107] Zhan X, Li D, Johns RA. Expression of endothelial nitric oxide synthase in ciliated epithelia of rats. J Histochem Cytochem 2003;51:81–7. doi:10.1177/002215540305100110.

[108] Rosselli M, Imthurn B, Macas E, Keller PJ. Endothelin production by bovine oviduct epithelial cells. J Reprod Fertil 1994;101:27–30.

[109] Rosselli M, Imthurn B, Macas E, Keller PJ, Dubey RK. Endogenous nitric oxide modulates endothelin-1 induced contraction of bovine oviduct. Biochem Biophys Res Commun 1994;201:143–8. doi:10.1006/bbrc.1994.1680.

[110] Jablonka-Shariff A, Olson LM. Nitric oxide is essential for optimal meiotic maturation of murine cumulus-oocyte complexes in vitro. Mol Reprod Dev 2000;55:412–21. doi:10.1002/(SICI)1098-2795(200004)55:4<412::AID-MRD9>3.0.CO;2-W.

[111] Nakamura Y, Yamagata Y, Sugino N, Takayama H, Kato H. Nitric Oxide Inhibits Oocyte Meiotic Maturation. Biol Reprod 2002;67:1588–92. doi:10.1095/biolreprod.102.005264.

[112] Dave S, Farrance DP, Whitehead SA. Evidence that nitric oxide inhibits steroidogenesis in cultured rat granulosa cells. Clin Sci (Lond) 1997;92:277–84.

[113] Romero-Aguirregomezcorta J, Santa ÁP, García-Vázquez FA, Coy P, Matás C. Nitric oxide synthase (NOS) inhibition during porcine in vitro maturation modifies oocyte protein S-nitrosylation and in vitro fertilization. PLoS One 2014;9:e115044. doi:10.1371/journal.pone.0115044.

[114] Goud AP, Goud PT, Diamond MP, Abu-Soud HM. Nitric Oxide Delays Oocyte Aging. Biochemistry 2005;44:11361–8. doi:10.1021/bi050711f.

[115] Tiwari M, Prasad S, Pandey AN, Premkumar K V, Tripathi A, Gupta A, et al. Nitric oxide signaling during meiotic cell cycle regulation in mammalian oocytes. Front Biosci (Schol Ed) 2017;9:307–18.

[116] Battaglia C, Ciotti P, Notarangelo L, Fratto R, Facchinetti F, de Aloysio D. Embryonic production of nitric oxide and its role in implantation: a pilot study. J Assist Reprod Genet 2003;20:449–54.

[117] Barroso RP, Osuamkpe C, Nagamani M, Yallampalli C. Nitric oxide inhibits development of embryos and implantation in mice. Mol Hum Reprod 1998;4:503–7. doi:10.1093/molehr/4.5.503.

[118] Manau D, Balasch J, Jiménez W, Fábregues F, Civico S, Casamitjana R, et al. Follicular fluid concentrations of adrenomedullin, vascular endothelial growth factor and nitric oxide in IVF cycles: relationship to ovarian response. Hum Reprod 2000;15:1295–9.

[119] Cameron IT, Campbell S. Nitric oxide in the endometrium. Hum Reprod Update 1998;4:565–9. doi:10.1093/humupd/4.5.565.

[120] Yallampalli C, Izumi H, Byam-Smith M, Garfield RE. An L-arginine-nitric oxide-cyclic guanosine monophosphate system exists in the uterus and inhibits contractility during pregnancy. Am J Obstet Gynecol 1994;170:175–85.

[121] Myatt L, Brockman DE, Eis AL, Pollock JS. Immunohistochemical localization of nitric oxide synthase in the human placenta. Placenta 1993;14:487–95.

[122] Conrad KP, Vill M, McGuire PG, Dail WG, Davis AK. Expression of nitric oxide synthase by syncytiotrophoblast in human placental villi. FASEB J 1993;7:1269–76. doi:10.1096/fasebj.7.13.7691671.

[123] Eis AL, Brockman DE, Pollock JS, Myatt L. Immunohistochemical localization of endothelial nitric oxide synthase in human villous and extravillous trophoblast populations and expression during syncytiotrophoblast formation in vitro. Placenta 1995;16:113–26.

[124] Norman JE, Cameron IT. Nitric oxide in the human uterus. Rev Reprod 1996;1:61–8.

OBJECTIVES

OBJECTIVES

The main objectives of this thesis were to study how NO modulates sperm capacitation in two different species, namely boar (in **Chapter 1**) and human (in **Chapter 2**) and determine whether or not the NO levels in the FF can predict the clinical outcomes from Assisted Reproduction Techniques (in **Chapter 3**).

To achieve the main objectives, the following specific objectives were established:

CHAPTER 1. Regulation of boar sperm functionality by the Nitric Oxide Synthase/Nitric Oxide system.

Objective 1. Effects of NO on boar sperm capacitation.

1.1. Description of the localization of NOS.

1.2. Analysis of sperm function in the presence/absence of a NO donor and NOS inhibitors. The following parameters were investigated: phosphorylation of serine, threonine and tyrosine residues, motility, acrosome reaction, phosphatidylserine translocation, intracellular calcium concentration.

Objective 2. Impact of nitric oxide on IVF. Three experimental conditions were analyzed, as follows:

2.1. Sperm co-incubated with cumulus-oocyte complexes.

2.2. Sperm co-incubated with decumulated oocytes.

2.3. Sperm co-incubated with decumulated oocytes and lower concentrations of the NO donor and NOS inhibitors.

CHAPTER 2. Nitric oxide-targeted protein phosphorylation during human sperm capacitation.

Objective 1. Effects of NO on protein phosphorylation in human spermatozoa. Sperm were treated or not with a NO donor and NOS inhibitors. Furthermore, the capacitation media was supplemented or not with L-Arginine, the substrate for nitric oxide synthesis, and FF. The regulation of sperm functionality was investigated by analyzing the phosphorylation of PKA substrates and tyrosine residues.

Objective 2. Mass spectrometry-based protein identification in specific bands (~ 110, ~ 87, ~ 75, ~ 62 kD), which showed different patterns of phosphorylation between the treatments.

CHAPTER 3. Follicular fluid nitrite and nitrate levels in oocyte donors: correlation with ovarian response.

Objective 1. Measurement of the FF levels of NO_2 and NO_3 via HPLC-UV/VIS in healthy women participating in an oocyte donation program.

Objective 2. Description of the association between the parameters mentioned above and the ovarian response to stimulation in these women.

CHAPTERS

CHAPTER 1

Regulation of boar sperm functionality by the Nitric Oxide Synthase/Nitric Oxide system

ABSTRACT

NO is a free radical synthesized mainly by NOS. This gas regulates many aspects in sperm physiology in different species. However, *in vitro* studies investigating NOS distribution, how NO influences sperm capacitation and IVF in porcine, have been lacking. In this sense, two main experiments were conducted: i) boar spermatozoa were capacitated in the presence/absence of S-Nitrosoglutathione (GSNO), a NO donor, and two NOS inhibitors, N^G-Nitro-L-Arginine Methyl Ester Hydrochloride (L-NAME) and Aminoguanidine Hemisulfate salt (AG); ii) IVF was performed in the presence or not of these supplements, but neither the oocytes nor the sperm were previously incubated in the supplemented media. Our results suggest that NOS distribution could be connected to pathways which lead to capacitation. Treatments showed significant differences after 30 min of incubation, compared to time zero in almost all motility parameters ($P<0.05$). When NOS were inhibited, three PKA substrates (~75, ~55 and ~50 kDa) showed lower phosphorylation levels between treatments ($P<0.05$). No differences were observed in total tyrosine phosphorylation levels evaluated by WB nor *in situ*. The percentage of acrosome-reacted sperm and phosphatidylserine translocation were significantly lower with L-NAME. Both inhibitors reduced sperm intracellular calcium concentration and IVF parameters, but L-NAME impaired sperm ability to penetrate denuded oocytes. These findings point out to the importance of both sperm and cumulus-oocyte derived NO in the IVF outcome in porcine.

1. INTRODUCTION

Several ROS, including hydrogen peroxide, superoxide anion and NO, have been shown to be involved in processes important for sperm physiology. Under normal, tightly regulated physiologic conditions, these ROS are essential for the sperm to acquire the fertilizing ability [1]. At physiologic levels, NO has been demonstrated to modulate sperm capacitation and acrosome reaction, sperm motility, and it may also have an anti-apoptotic effect (reviewed by [2]). Besides, the importance of NO in oocyte maturation and subsequent fertilization has also been revealed [3].

It is known that sperm can produce NO, but the evidence that the endogenous synthesis is sufficient to be physiologically significant is equivocal [4]. Various cell types in the mammalian female reproductive tract generate substantial levels of NO, which in turn determine the S-nitrosylation of sperm proteins. Thus, *in vivo* is more likely to occur a response to the NO generated by the female tract cells, rather than by autocrine effects of sperm-generated NO. Furthermore, it has been demonstrated that NOS activity, the enzymes responsible for NO synthesis, can be modulated by sexual hormones [5], therefore, the NO levels will vary during the oestrus cycle [6] which in turn could regulate the fertilization process.

Sengoku *et al*. [7] showed that low concentrations of NO may have a physiologic role in fertilization by enhancing the capacitation and ZP binding, but not by inducing the acrosome reaction or facilitating oocyte penetration. On the other hand, Herrero *et al*. [8] showed that the incubation of spermatozoa with NOS inhibitors reduced the IVF outcome in mouse. These authors observed that NOS inhibition during sperm capacitation impaired the spontaneous acrosome reaction, as well as the IVF. However, studies on the production of certain substances during the interaction of gametes that affect IVF performance have been scarce. In this sense, it has been described that both spermatozoa and cumulus cells produce NO and this molecule takes part in the fertilization process [3,9]. Nevertheless, despite all the studies carried out to determine the role of NO on sperm function, we should improve our understanding of how this gas modulates it by performing tests that bring us closer to the physiological conditions during fertilization. In relation to the studies using human spermatozoa and their interaction with the female gamete, it has only been possible to analyze hemizone binding assays [7,10], logically for ethical reasons. On the other hand, IVF assays performed in mouse were done with epididymal spermatozoa which cannot be considered physiologically mature. Therefore, these studies, despite the important information they provide, cannot be considered conclusive.

It appears that while NO synthesis in sperm is required for IVF, the free radicals generated in the medium, including NO, could be in excess and be harmful, as seen in certain infertility cases [11]. In

porcine, they could affect the functionality of both spermatozoa and oocytes and, somehow, contribute to the problem of polyspermy (i.e. fertilization of an ovum by more than one spermatozoon) in this species. However, polyspermy could be used as a tool to evaluate sperm functionality since a higher percentage of penetrated oocytes and sperm number per penetrated oocyte correlate with sperm quality [12].

For all the reasons above, this paper aims to determine the role of the NOS/NO system in the fertilizing capacity of boar spermatozoa. Besides, since the NO function during the fertilization process in porcine has not yet been determined, we hypothesized that by regulating the NOS/NO system, the IVF efficiency could be improved. To develop this hypothesis, we determined, at first, the NO effects on the spermatozoon, followed by its impact on the IVF.

2. MATERIALS AND METHODS

Ethics

The study was carried out following the Spanish Policy for Animal Protection RD 53/2013, which meets European Union Directive 2010/63/UE on animal protection. The Ethics Committee of Animal Experimentation of the University of Murcia and the Animal Production Service of the Agriculture Department of the Region of Murcia (Spain) (ref. no. A13160609) approved the procedures performed in this work.

Materials

Unless otherwise stated, chemicals and reagents were purchased from Sigma-Aldrich Química S.A. (Madrid, Spain). Equine chorionic gonadotropin (eCG; Foligon) was supplied by Intervet International BV (Boxmeer, Holland), human chorionic gonadotropin (hCG; VeterinCorion) by Divasa Farmavic (Barcelona, Spain) and Percoll by GE Healthcare (Uppsala, Sweden). The prolonged antifade mounting medium (SlowFadeAntifade Kit) was obtained from Invitrogen (Paisley, United Kingdom). N^G-nitro-L-Arginine methyl ester (L-NAME; 483125) was purchased from Calbiochem (distributed by Merck Chemicals, Beeston, Nottingham, UK).

Culture media

IVM of pig oocytes was carried out using the NCSU-37 medium [13] supplemented with 0.57 mM cysteine, 1 mM dibutyryl cAMP, 5 mg/mL insulin, 50 μM β-mercaptoethanol, 10 IU/mL eCG, 10 IU/mL hCG and 10% *v/v* porcine FF.

Sperm capacitation and IVF were performed using TALP medium [14], consisting of 114.06 mM NaCl, 3.2 mM KCl, 8 mM Ca^{2+}-Lactate.$5H_2O$, 0.5 mM $MgCl_2.6H_2O$, 0.35 mM NaH_2PO_4, 25.07 mM $NaHCO_3$, 10 mM Na-lactate, 1.1 mM Na-pyruvate, 5 mM glucose, 2 mM caffeine, 3 mg/mL bovine serum albumin (BSA, A-9647), 1 mg/mL polyvinyl alcohol (PVA) and 0.17 mM kanamycin sulfate.

Sperm collection

Sperm samples were collected from boars with proven fertility by the gloved hand method. Standard laboratory techniques were applied to evaluate sperm concentration, motility, acrosome integrity, and normal morphology.

Immunocytochemistry: NOS detection and Tyrosine Phosphorylation (Tyr-P) by Indirect Immunofluorescence (IIF)

To determine NOS localization, a method adapted from Meiser and Schulz [15] was used. Briefly, ejaculated boar sperm were washed with Dulbecco's Phosphate-Buffered Saline without calcium chloride and magnesium chloride (DPBS) and spread on glass slides coated with poly L-lysine. Spermatozoa were air-dried and fixed for 20 min in ice-cold 3% *v/v* paraformaldehyde in DPBS containing 120 mM sucrose. They were gently rinsed with DPBS, incubated for 10 min in ice-cold 100% *v/v* methanol, and triply washed with DPBS. Specimens were treated with blocking I solution (10% *w/v* BSA, 1% *v/v* Triton X-100, dissolved in distilled water, 1 h, 20 °C). Next, sperm were incubated with blocking II solution (2% *w/v* BSA, 1% *v/v* Triton X-100, dissolved in distilled water, 1 h, 37 °C), which included the primary anti-NOS antibodies (all three produced in mouse, 1:1000): anti-nNOS [N2280, monoclonal, clone NOS-B1, obtained with a recombinant nNOS fragment (amino acids 1-181) from rat brain], anti-eNOS [N9532, monoclonal, clone NOS-E1, obtained with a synthetic peptide corresponding to bovine eNOS (amino acids 1185-1205 with an N-terminally added lysine) conjugated to KLH] or anti-iNOS [N9657, monoclonal, clone NOS-IN, obtained with a synthetic peptide corresponding to iNOS from mouse macrophage (amino acids 1126-1144) conjugated to KLH]. These anti-NOS antibodies were chosen since their reactivity with porcine sperm extracts was previously shown by Aquila *et al*. [16]. Then, the specimens were triply washed with blocking II and probed overnight (4 °C) with a FITC-labelled secondary antibody (goat anti-mouse, 1:1000, diluted in blocking II). For controls, specimens were processed in the absence of primary and/or secondary antibody.

Tyr-P location was studied as previously described [17], using an anti-phosphotyrosine antibody (4G10, Millipore, CA, USA, 1:300 in 1% *w/v* BSA). The secondary antibody was a fluorescein-conjugated goat anti-mouse (Bio-Rad Laboratories, Madrid, Spain, 1:400 in 1% *w/v* BSA).

All images were taken at x1000 (for NOS distribution) and x400 (for Tyr-P location) magnifications, using the AxioVision imaging system (Rel. 4.8) with an AxioCamHRc camera (Carl Zeiss, Göttingen, Germany) attached to a Leica DMR fluorescence microscope (Leica Microsystems, Wetzlar, Germany) equipped with fluorescent optics (blue filter, BP 480/40; emission BP 527/30).

Spermatozoa motion assay

To evaluate sperm motility, computer-assisted sperm analysis (CASA) was performed (ISAS® system, PROISER R+D S.L., Valencia, Spain) and the following parameters were studied: total motility (%), progressive motility (%), curvilinear velocity (VCL, μm/s), straight-line velocity (VSL, μm/s), average path velocity (VAP, μm/s), linearity of the curvilinear trajectory (LIN, ratio of VSL/VCL, %), straightness (STR, ratio of VSL/VAP, %), amplitude of lateral head displacement (ALH, μm), wobble of the curvilinear trajectory (WOB, ratio of VAP/VCL, %), and beat cross-frequency (BCF, Hz). For this purpose, a 4 μL drop of the sample was placed on a warmed (38.5 °C) Spermtrack ST20 chamber (PROISER R+D S.L) and analyzed using a phase-contrast microscope (x200 magnification; Leica DMR, Wetzlar, Germany). The setting parameters were: 60 frames at 30 frames/s, of which spermatozoa had to be present in at least 15 to be counted. Spermatozoa with a VCL less than 10 μm/s were considered immotile. A minimum of five fields per sample were evaluated, counting a minimum of 200 spermatozoa per field.

Western Blotting (WB): Protein Kinase A substrates (PKAs-P) and Tyr-P

Sperm protein extracts were isolated from 1×10^6 spermatozoa/sample and immunoblotted as described by Navarrete *et al*. [18] with the following antibodies: anti-phospho-PKA substrates (9624, Cell Signaling Technology, Beverly, USA, 1:2000), anti-phosphotyrosine (4G10, Millipore, CA, USA, 1:10000) and anti-β-tubulin (T0198, Sigma-Aldrich®, Madrid, Spain, 1:5000). The Pierce® ECL 2 Western Blotting Substrate (80196, Lumigen Inc, Southfield, MI, USA) coupled with a chemiluminescence system (Amersham Imager 600, GE Healthcare Life Sciences, Buckinghamshire, UK) were used to visualize the blots. The relative amount of signal in each membrane was quantified using the ImageQuant TL v8.1 software (GE Healthcare).

Acrosome Reaction (AR) assay

Boar spermatozoa were capacitated for 1 h, subsequently exposed for 30 min to 3 ng/mL progesterone under the different experimental conditions, after which the percentage of acrosome-reacted sperm was evaluated by staining with FITC-conjugated peanut agglutinin from Arachis hypogaea (PNA-FITC L7381, Sigma-Aldrich®, Madrid, Spain), as previously described [19]. Samples were analyzed under an epifluorescence microscope at x400 magnification.

Detection of membrane phosphatidylserine (PS) translocation

Translocation of PS residues to the outer leaflet of the plasma membrane was detected with an Annexin V-Cy3™ Apoptosis Detection Kit (Sigma, Madrid, Spain). For this assay, 1 µL Annexin V with 5 µL 6-carboxyfluorescein diacetate (6-CFDA) in 450 µL of binding buffer (commercial kit) were mixed with 50 µL of each sperm sample. After 10 min of incubation in the dark, at room temperature, samples were fixed with 10 µL formaldehyde (10% *v/v* in DPBS). Each sample was placed on a slide and examined at x400 magnification by epifluorescence microscopy. Viable sperm (6-CFDA+) were visualized in green with a standard fluorescein filter and Annexin+ sperm (labelling PS exposure, Annexin V-Cy3.18+) in red (N2.1 filter; excitation BP 515–560 nm) [20].

Determination of intracellular calcium concentration ($[Ca^{2+}]i$)

$[Ca^{2+}]$i was measured according to a method reported previously [21,22]. Specifically, spermatozoa were incubated with 2.5 µM Fura-2/AM in a buffer medium consisting of 2.7 mM KCl, 1.5 mM KH_2PO_4, 8.1 mM Na_2HPO_4, 137 mM NaCl, 5.55 mM glucose, and 1 mM pyruvate for 45 min at 37 °C. The extracellular unloaded Fura-2 was removed by centrifugation (700 × g, 5 min). Washed sperm were resuspended in the same buffer to a concentration of 3×10^8 cells/mL and incubated at 37 °C for 15 min in the dark. Then, spermatozoa were centrifuged (700 × g, 5 min) and resuspended in TALP medium. As a negative control, spermatozoa were also resuspended in DPBS. Fluorescence was monitored using the Jasco FP-6300 spectrofluorimeter (Jasco, Madrid, Spain) for a further 30 min. Excitation wavelengths alternated between 340 and 380 nm with emission held at 510 nm. At the end of the experiments, sperm were lysed with 0.5% *v/v* Triton X-100, and then Ca^{2+} was depleted by addition of 25 mM EGTA. $[Ca^{2+}]$i was calculated as previously described [23]. For the statistical analysis, the Ca^{2+} concentration (nM/L) was recorded from 0 to 1800s at 30s intervals for every experimental group and replicate. Finally, the mean value during the incubation period was calculated.

Oocyte collection and IVM

Ovaries from Landrace by Large White gilts were collected at a local slaughterhouse (El Pozo Alimentación S.A., Alhama de Murcia, Murcia, Spain) and transported within 30 min after slaughter to the laboratory in saline solution containing 100 µg/mL kanamycin sulfate at 38.5 °C. Before collecting the cumulus-oocyte complexes (COCs), ovaries were washed once in 0.04% *w/v* cetrimide solution and twice in saline. COCs from antral follicles (3–6 mm diameter) were washed twice with DPBS supplemented with 1 mg/mL PVA and 0.005 mg/mL red phenol, and twice more in maturation medium previously equilibrated for a minimum of 3 h at 38.5 °C under 5% CO_2 in air. Groups of 50

COCs with complete and dense cumuli oophori were cultured in 500 µL maturation medium for 22 h at 38.5 °C under 5% CO_2 in air. Following this incubation, COCs were washed twice in fresh maturation medium without dibutyryl cAMP, eCG, and hCG and cultured for an additional period of 20–22 h.

IVF and zygote staining

Following the 44 h culture in maturation medium, COCs were stripped or not (see the experimental design) of cumulus cells by pipetting and then washed three times with TALP medium. The IVF medium was previously equilibrated at 38.5 °C under 5% CO_2 in a 4-well dish (250 µL/well) and groups of 50 oocytes were transferred into each well. Semen aliquots (0.5 mL) from different boars were mixed and subjected to a discontinuous Percoll gradient (45 and 90% *v/v*, 740 × g, 30 min). The resultant sperm pellets were diluted in TALP medium and centrifuged again (10 min at 740 × g). After diluting the pellet again in TALP, an aliquot of this suspension was used for IVF, giving a final concentration of 2.5×10^5 spermatozoa/mL or 2.5×10^4 spermatozoa/mL, depending on the experiment. The medium was supplemented with NOS inhibitors, NO donor or not supplemented, as described in the experimental design. At 18–20 h post-insemination, putative zygotes were fixed and stained for evaluation as previously described [3].

Statistical analysis

The data are presented as the mean ± standard error of the mean (SEM) and were tested for normality using the Kolmogorov-Smirnov test, and the homogeneity of variance was determined using the Levene test. ANOVA was used for the statistical analysis and the means were separated using the Tukey test at $P<0.05$. Since the data regarding the acrosome reaction experiment did not satisfy the Kolmogorov-Smirnov and Levene tests, the Kruskal-Wallis test was applied, and treatment average ranks were separated using the stepwise step-down multiple comparisons method [24] at $P<0.05$. The true means of the data, rather than ranked means, are presented. All statistical analyses were conducted using IBM SPSS Statistics for Windows, Version 20.0 (IBM, Armonk, NY, USA).

Experimental design

Experiment 1: Effects of NO on sperm capacitation

To investigate how the NOS/NO system regulates sperm functionality (Fig. 1: Experiment 1), sperm samples were incubated in TALP medium for 60 minutes at 38.5 °C and 5% CO_2 with different treatments. Four experimental groups were established according to the treatment used: CONTROL: spermatozoa incubated in the absence of any treatment; GSNO: spermatozoa incubated in the

presence of 100 μM S-Nitrosoglutathione; L-NAME: spermatozoa incubated in the presence of 10 mM N^G-Nitro-L-Arginine Methyl Ester Hydrochloride; AG: spermatozoa incubated in the presence of 10 mM Aminoguanidine Hemisulfate salt. These concentrations were chosen based on a literature review [3,4,25].

The experimental groups mentioned above were subjected to the following tests: IIF (to determine NOS localization and Tyr-P *in situ*), WB (to evaluate the phosphorylation of PKA substrates and Tyr-P), AR assay, PS translocation assay and measurement of $[Ca^{2+}]i$. However, to avoid sperm agglutination, which hinders cell detection by CASA systems, and since previous studies have reported 30 min sperm incubation under capacitation conditions were sufficient to observe changes in sperm motility parameters [26], this period of time was considered to be suitable to assess the effect of the NOS/NO system on sperm motion.

Experiment 2: Impact of NO on IVF

To assess how the NOS/NO system modulates the IVF in porcine species, three experiments were performed (Fig. 1: Experiment 2A, B and C). All experiments were started using *in vitro* matured oocytes and IVF was performed by adding to the medium the above-mentioned NO donor and NOS inhibitors. As a control group, IVF was performed in the absence of any treatments. The spermatozoa employed during IVF were not previously treated with these supplements. The percentage of sperm penetration, the sperm number per oocyte, the number of sperm bound to the ZP, as well as the percentage of male pronucleus formation were determined in all experiments.

Experiment 2A: Effects of NO on the interaction between spermatozoa and COCs

IVF was performed using COCs that were co-incubated with 2.5×10^5 spermatozoa/mL. The GSNO was used at a concentration of 100 μM, whereas for the NOS inhibitors (L-NAME and AG) the concentration was 10 mM. This experiment was repeated five times and a total of 549 oocytes were evaluated.

Experiment 2B: Effects of NO on the interaction between spermatozoa and decumulated oocytes

Since the cumulus cells also produce NO [27], this second experiment was performed to investigate how the presence/absence of NO alters the interaction between sperm and decumulated oocytes. IVF was performed using the same concentrations of NO donor and NOS inhibitors as in experiment A. This experiment was repeated three times and a total of 258 oocytes were evaluated.

Experiment 2C: Effects of low NOS inhibitors concentration on the interaction between spermatozoa and decumulated oocytes

The latter assay was developed to evaluate whether there is a dose-dependent effect of the NO donor and NOS inhibitors. For this, IVF was performed using decumulated oocytes, 2.5×10^4 spermatozoa/mL and a lower concentration of NO donor and NOS inhibitors (50 µM GSNO and 5 mM for the inhibitors, respectively). This experiment was repeated three times and a total of 351 oocytes were evaluated.

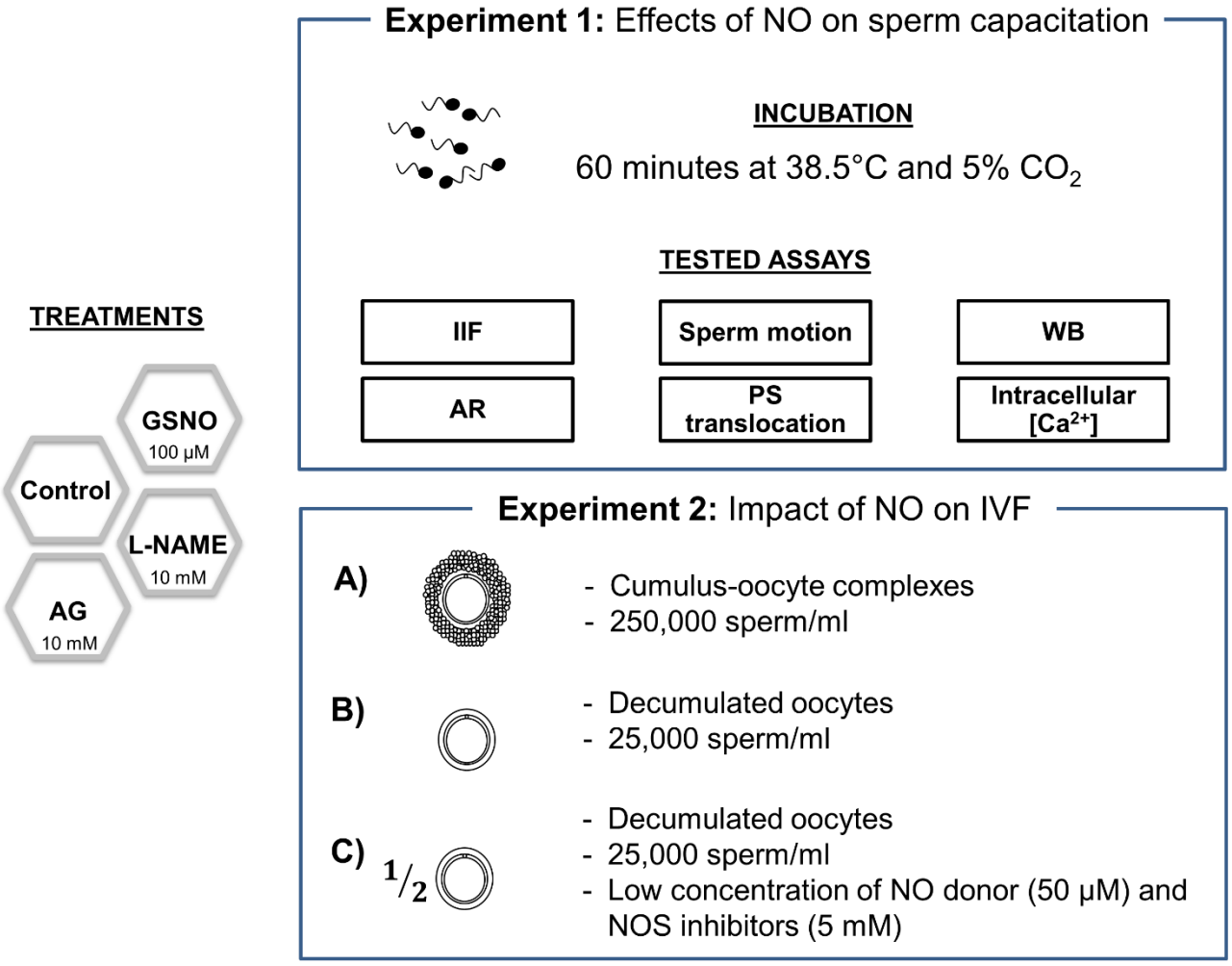


Figure 1. Analysis of the effects of a NO donor and two NOS inhibitors on sperm capacitation and *in vitro* fertilization. Experimental design. *Experiment 1*: Spermatozoa were incubated for 60 minutes in presence or not of these supplements. After that, the following assays were used to evaluate the sperm capacitation status: indirect immunofluorescence (IIF), motility assay, Western Blotting (WB), acrosome reaction (AR), phosphatidylserine translocation (PS) and measurement of the intracellular calcium concentration; *Experiment 2*: The *in vitro* fertilization (IVF) was performed in the presence or not of the NO donor and NOS inhibitors, under three circumstances: (**A**) Intact cumulus-oocyte complexes and a sperm concentration of 250,000 spermatozoa/mL. (**B**) Decumulated oocytes and a sperm concentration of 25,000

spermatozoa/mL. (**C**). Lower concentrations of the NO donor, NOS inhibitors and spermatozoa. Neither the oocytes nor the spermatozoa were treated with the NO donor or NOS inhibitors before performing the IVF.

3. RESULTS

3.1. Experiment 1: Effects of NO on sperm capacitation

3.1.1. NOS localization

The three isoforms of NOS, neuronal (nNOS), endothelial (eNOS) and inducible NOS (iNOS) have been identified in different mammalian spermatozoa, including the boar [16]. However, to our knowledge no study has been performed to localize NOS in porcine ejaculated spermatozoa. Therefore, we used IIF to identify the distribution of these enzymes.

The eNOS was identified in the acrosomal region, although a weak fluorescent signal was also registered in the principal and end piece of the flagellum (Fig. 2). Similarly, the nNOS-associated fluorescence was concentrated in the sperm head region, with a lower fluorescence in the principal and end piece of the flagellum (Fig. 2). Moreover, immunofluorescent iNOS-staining was spread over the acrosomal, postacrosomal and neck region, but also in the principal and end piece of the tail (Fig 2).

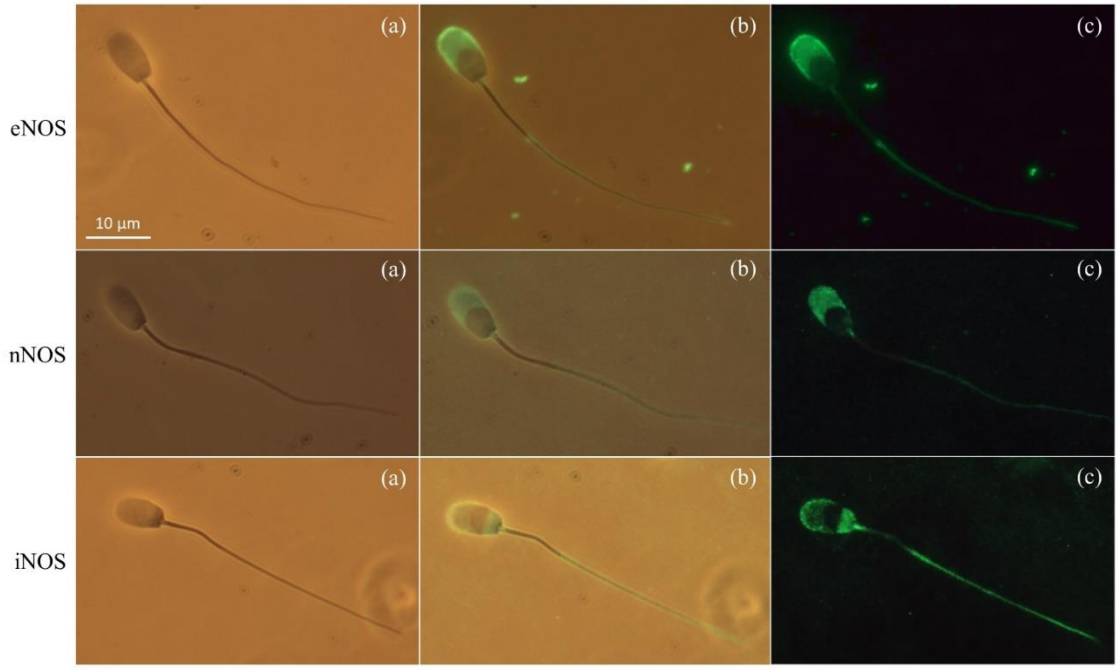


Figure 2. Localization of NOS isoforms by indirect immunofluorescence. Spermatozoa were fixed, permeabilized, incubated with specific anti-eNOS, nNOS and iNOS primary antibodies, together with a FITC-labelled secondary antibody and examined under an epifluorescence microscope at x1000 magnification. Representative pictures are shown by phase-contrast microscopy (**a**), merging the phase-contrast image with

the green fluorescence pattern (**b**) and for the immunofluorescent staining (**c**). The eNOS- and nNOS-associated fluorescence were identified in the sperm head region, with a lower staining in the principal and end piece of the tail. The iNOS staining pattern was spread over the acrosomal, postacrosomal and neck region, but also in the principal and end piece of the flagellum.

3.1.2. Motility parameters

The role of NO in sperm motility is controversial, with studies suggesting both a beneficial [28,29] or a detrimental effect [30,31].

When the CASA evaluation was performed in the present study, at 0 min incubation time (Table 1) none of the motility parameters showed statistical differences ($P>0.05$). Later, at 30 min of incubation (Table 1), no differences were found for Total motility, Progressive motility, VCL, LIN, STR, WOB, ALH or BCF. However, when the VSL was studied, CONTROL and GSNO groups (17.2 ± 1.3 and 17.9 ± 1.8; respectively) were found to be significantly different from AG (11.8 ± 0.8) but no differences were observed with L-NAME (16.4 ± 0.5). Continuing the sperm motion analysis, when we analyzed VAP at 30 mins, both CONTROL and GSNO showed the highest values (26.6 ± 1.8 and 26.9 ± 2.6, respectively), which did not differ from L-NAME group (25.5 ± 0.8) but were significantly different from AG (19.7 ± 0.9).

When looking at the effect of incubation time on the CASA parameters, we observed that at 30 min of incubation, all treatments showed significant differences compared to their values at time zero for Total Motility, Progressive Motility, VCL, VSL, VAP and BCF. Same difference was found among all treatments in ALH, except for GSNO. Finally, when we compared the values for LIN, STR and WOB at T=30 min, the different treatments did not statistically differ from their T=0 min counterparts.

Table 1. Effects of NO on sperm motility parameters at 0 and 30 minutes of incubation.

Incubation time	Treatment	*n*	Total motility	Progressive motility	VCL	VSL	VAP	LIN	STR	WOB	ALH	BCF
0 min	CONTROL	6	86.8 ± 2.8	84.0 ± 3.2	95.1 ± 11.3	38.2 ± 4.9	59.7 ± 4.7	29.3 ± 9.3	39.2 ± 12.4	48.3 ± 15.1	3.1 ± 0.4	8.6 ± 0.3
	GSNO	6	83.6 ± 1.3	79.0 ± 2.2	90.4 ± 15.4	35.2 ± 3.8	54.6 ± 4.9	29.0 ± 9.2	39.2 ± 12.4	47.3 ± 14.8	3.1 ± 0.6	8.7 ± 0.3
	L-NAME	6	86.7 ± 2.8	82.4 ± 3.7	99.3 ± 17.5	36.6 ± 5.6	59.2 ± 6.1	26.7 ± 8.7	35.7 ± 11.6	49.0 ± 15.4	3.4 ± 0.7	8.2 ± 0.4
	AG	6	81.8 ± 3.2	77.4 ± 4.0	92.6 ± 13.1	41.0 ± 7.3	59.8 ± 7.0	29.9 ± 9.7	40.5 ± 13.1	47.5 ± 14.9	3.0 ± 0.5	8.8 ± 0.5
30 min	CONTROL	6	36.9 ± 5.6*	26.7 ± 4.9*	49.5 ± 7.0*	17.2 ± 1.3^{a*}	26.6 ± 1.8^{a*}	28.7 ± 9.0	41.5 ± 13.0	42.3 ± 13.3	2.0 ± 0.3*	5.5 ± 0.2*
	GSNO	6	41.0 ± 6.8*	29.9 ± 5.8*	50.9 ± 8.1*	17.9 ± 1.8^{a*}	26.8 ± 2.6^{a*}	29.0 ± 9.4	42.6 ± 13.4	41.9 ± 13.3	2.1 ± 0.3	5.7 ± 0.3*
	L-NAME	6	45.9 ± 7.7*	32.2 ± 6.8*	43.8 ± 4.2*	16.4 ± 0.5^{a,b*}	25.5 ± 0.8^{a,b*}	29.9 ± 9.4	42.1 ± 13.2	45.7 ± 14.3	1.8 ± 0.2*	6.0 ± 0.2*
	AG	6	30.6 ± 4.0*	21.3 ± 3.2*	43.6 ± 4.2*	11.8 ± 0.8^{b*}	19.7 ± 0.9^{b*}	24.1 ± 8.1	40.5 ± 12.9	36.5 ± 11.7	1.9 ± 0.2*	5.2 ± 0.3*

n (number of replicates), Total motility (%), Progressive motility (%), VCL (curvilinear velocity, μm/s), VSL (straight-line velocity, μm/s), VAP (average path velocity, μm/s), LIN (linearity of the curvilinear trajectory, ratio of VSL/VCL, %), STR (straightness, ratio of VSL/VAP, %), WOB (wobble of the curvilinear trajectory, ratio of VAP/VCL, %), ALH (amplitude of lateral head displacement, μm), and BCF (beat cross-frequency, Hz). All data are expressed as the mean ± SEM. Different superscripts ^{a,b} within the same column indicate statistical significance ($P<0.05$), while * indicates statistical significance ($P<0.05$) throughout the incubation time.

3.1.3 Protein Kinase A substrates (PKAs-P) and Tyrosine Phosphorylation (Tyr-P)

The sperm capacitation process involves the early activation of protein kinases and the inactivation of protein phosphatases [32]. It has been reported that NO can modulate this process through the activation of the cAMP/PKA pathway [33] and it is directly involved in tyrosine phosphorylation by modulating both the cAMP/PKA and ERK pathways (reviewed by [34]).

To determine the effects of the NO donor and NOS inhibitors on boar sperm capacitation, PKAs-P and Tyr-P were analyzed and quantified by WB (Fig. 3).

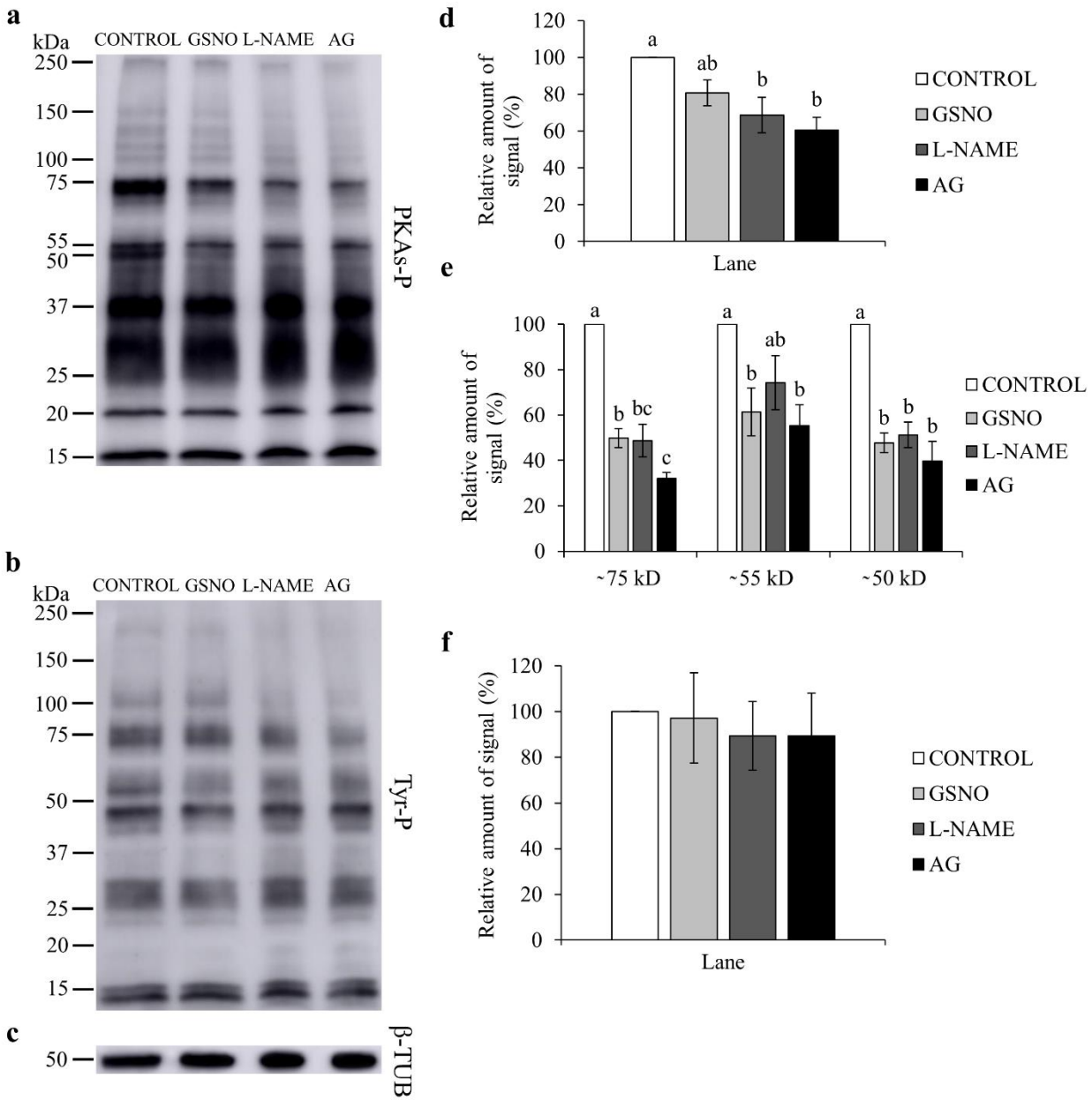


Figure 3. Effect of GSNO, L-NAME and AG on PKA substrates (PKAs-P) and Tyrosine phosphorylation (Tyr-P). Sperm were incubated for 60 min under capacitating conditions in the absence of

any treatments (CONTROL) or in the presence of GSNO, a NO donor, and L-NAME and AG (both NOS inhibitors). (**a** and **b**) Sperm protein extracts were analyzed for phosphorylation by Western blotting using anti-PKAs-P or anti-Tyr-P as first antibodies, respectively. (**c**) β-tubulin was used as a protein loading control. For signal quantification, each lane was normalized to its β-tubulin optical density value. (**d, e** and **f**) Relative amount of signal quantified in each membrane using ImageQuant TL v8.1 software for PKAs-P and Tyr-P, respectively. Different letters (a, b, c) indicate statistically significant differences ($P<0.05$) between groups.

Our results showed that the phosphorylation levels for PKAs-P were significantly lower when using the NOS inhibitors than in the CONTROL group (Figs. 3a and d), whereas the NO donor had no significant effect. Interestingly, the analysis of the relative optical density revealed the presence of three PKA substrate species of approximately 75, 55 and 50 kDa which seemed to possess a specific pattern of phosphorylation (Figs. 3a and e). In detail, the NO donor and NOS inhibitors lowered significantly the degree of phosphorylation in the ~75 and ~50 kDa species compared with their levels in the CONTROL ($P<0.05$), but in the ~55 kDa species this effect was evident only when the capacitation took place in the presence of GSNO and AG ($P<0.05$).

On the other hand, when considering the phosphorylation levels of tyrosine residues, no significant effects were observed in the presence of both the NO donor and NOS inhibitors (Figs. 3b and f).

3.1.4. Tyr-P detection by IIF

A crucial event involved in capacitation and the acquisition of fertilizing potential is protein Tyr-P [35]. Different sperm subpopulations were identified within a sample according to their degree of capacitation and hyperactivation (Table 2). No significant differences were found between groups with regard to the four Tyr-P patterns analyzed ($P>0.05$).

Table 2. Effects of NO on the immunolocalization of protein Tyr-P.

Treatment	*n*	Pattern I (%)	Pattern II (%)	Pattern III (%)	Pattern IV (%)
CONTROL	8	10.8 ± 1.9	60.8 ± 9.2	28.5 ± 9.4	63.7 ± 9.7
GSNO	8	11.2 ± 2.3	53.2 ± 9.9	35.9 ± 9.1	64.3 ± 10.9
L-NAME	8	20.0 ± 6.6	46.4 ± 11.3	33.6 ± 11.3	63.0 ± 10.3
AG	8	10.9 ± 2.8	49.4 ± 11.3	39.8 ± 10.9	64.6 ± 8.2

n (number of replicates); Pattern I, low capacitation status (non-phosphorylated or head- and/or flagellum-phosphorylated spermatozoa); Pattern II, medium capacitation status (equatorial segment or equatorial segment and flagellum-phosphorylated spermatozoa); Pattern III, high capacitation status (equatorial segment and head and/or flagellum-phosphorylated spermatozoa); Pattern IV, flagellum phosphorylation independent of phosphorylation in other locations.

3.1.5. AR assay

Progesterone is known to induce the acrosome reaction in capacitated sperm [36], so we determined how this process might be modulated by NO in boar spermatozoa. The results represented in Fig. 4 indicated that the GSNO and AG treatments did not influence the percentage of acrosome-reacted sperm when compared to the CONTROL. However, L-NAME reduced significantly this percentage ($P<0.05$).

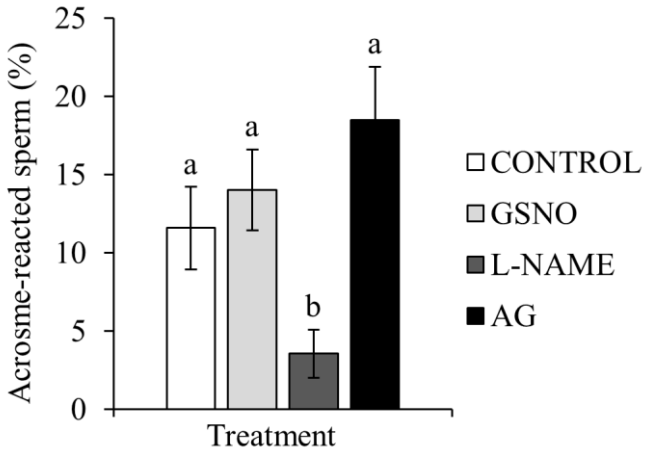


Figure 4. Effect of GSNO, L-NAME and AG on the acrosome reaction. After being incubated in capacitating conditions for 60 min in the absence of any treatments (CONTROL) or in the presence of GSNO, a NO donor, and L-NAME and AG (both NOS inhibitors), the sperm were exposed to 3 ng/mL progesterone during another 30 min under the different experimental conditions. Next, the percentage of acrosome-reacted sperm was evaluated by PNA-FITC staining. Different letters (a, b) indicate statistically significant differences ($P<0.05$) between groups.

3.1.6. PS translocation

In boar spermatozoa, the capacitating agents had been shown to induce rapid changes in the membrane lipid architecture such as the external exposure of PS, which is also commonly recognized as a marker of apoptosis [37,38]. As is considered that NO participates in both processes, we decided to investigate the involvement of NO in the PS translocation during sperm capacitation.

The results (Fig. 5) showed that the NO donor had no significant effect on PS externalization. In fact, both the CONTROL and the GSNO groups reached similar levels of PS translocation (37.67% and 38%, respectively). On the other hand, when using the NOS inhibitors, a significant difference was observed only with L-NAME which had a lower PS level than both the GSNO and CONTROL groups (29.83%; $P<0.05$). Sperm viability was higher than 50% in all the treatments (data not shown).

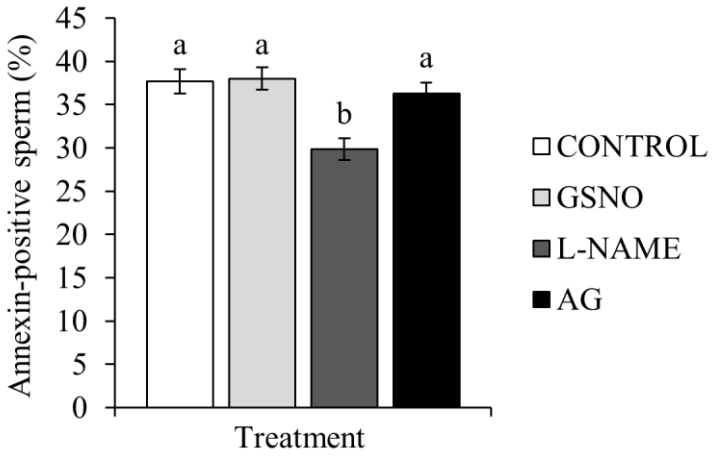


Figure 5. Effect of GSNO, L-NAME and AG on PS translocation. Following incubation under capacitating conditions, the translocation of PS residues was analyzed with an Annexin V-Cy3™ Apoptosis Detection Kit. Different letters (a, b) indicate statistically significant differences ($P<0.05$) between groups.

3.1.7. Determination of $[Ca^{2+}]i$

The regulation of Ca^{2+} is a fundamental step during the capacitation process [39], therefore, we monitored its levels before and after our treatments (Fig. 6). During the period prior to the addition of treatments (600 s), Ca^{2+} intake increased throughout the incubation time. Treatment with GSNO did not affect $[Ca^{2+}]i$ vs CONTROL. However, both inhibitors had an effect on the spermatozoa, in fact results showed that L-NAME reduces abruptly the $[Ca^{2+}]i$ at a basal level, while with AG the reduction is more gradual after its addition.

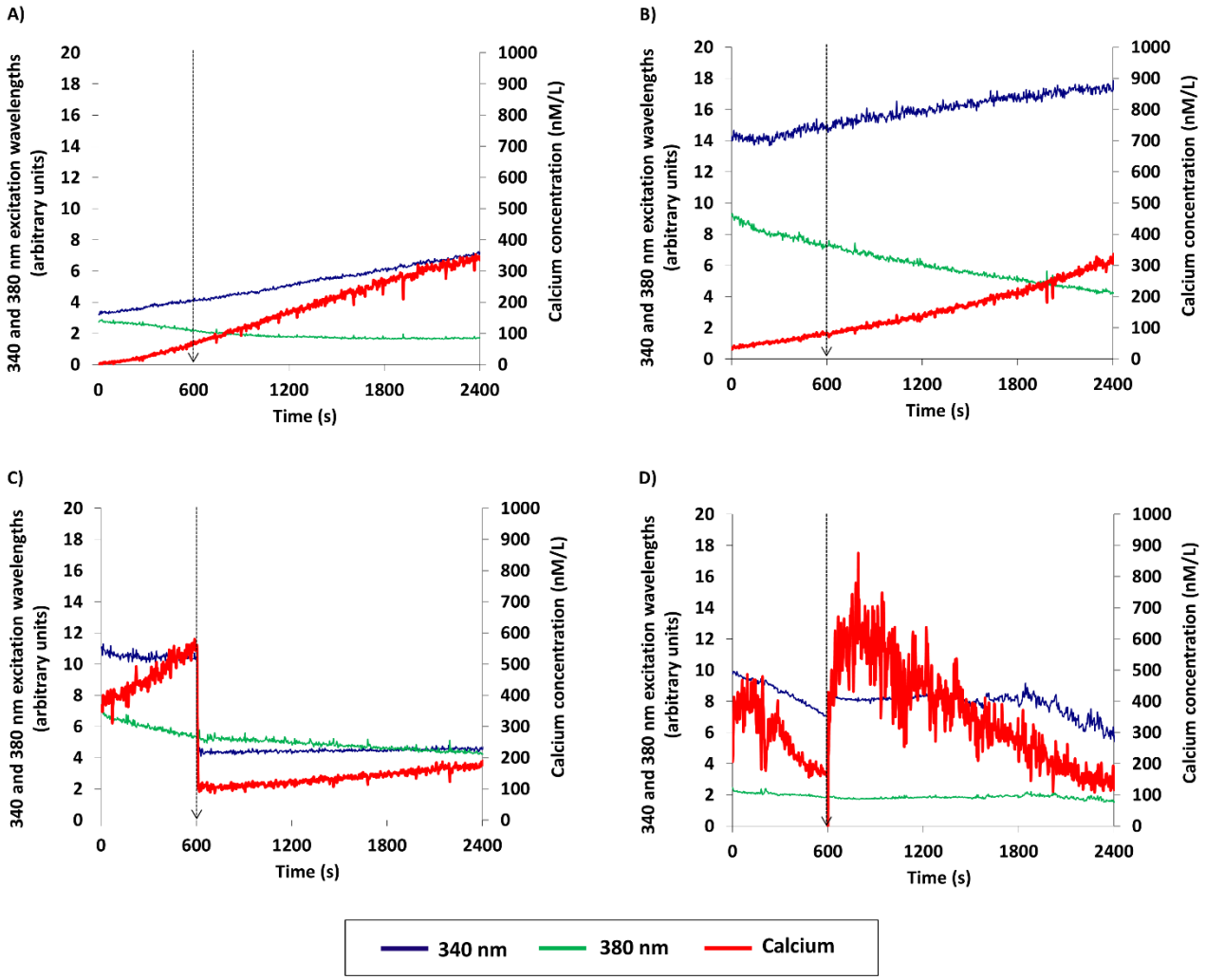


Figure 6. Intracellular calcium concentration. Graphs show the measurements collected from the different treatments: **A)** Control, **B)** GSNO, **C)** L-NAME and **D)** AG. The excitation wavelengths are shown with blue (340 nm) and green (380 nm) lines, while the intracellular calcium concentration is shown by the red line. Fluorescence was measured with the calcium indicator Fura-2/AM and monitored using a spectrofluorimeter for 40 min. The system was stabilized for 10 min (dashed arrow) before adding or not the treatment.

3.2. Experiment 2: Impact of NO on IVF

NO is one of the components of the environment where fertilization occurs and is generated by oviductal cells [40,41], oocytes and cumulus cells [9,42], but also spermatozoa [15,43,44]. Besides, NO is necessary for sperm capacitation to occur [45]. However, it has been suggested that the sperm NO production is low and most likely these cells encounter sufficient NO levels to support capacitation inside the female genital tract [46]. For these reasons, we studied the effects of NO on the IVF parameters with and without cumulus cells.

3.2.1. Experiment 2A: Effects of NO on the interaction between spermatozoa and COCs

The results (Table 3) showed that the inhibition of NO production affected all IVF parameters. The percentage of oocytes that had been fertilized in the presence of inhibitors decreased. The AG inhibitor reduced the IVF parameters, but these were higher than L-NAME group. As for the number of spermatozoa bound to the ZP and the mean number of spermatozoa per oocyte, we observed that they decreased both with the use of GSNO and with NOS inhibitors. In all the parameters analyzed, the NOS inhibitor with the greatest effect was L-NAME.

Table 3. Effects of NO during IVF with intact cumulus-oocyte complexes.

Treatment	*n*	Penetration (%)	Sperm/Oocyte (n)	Sperm/ZP (n)	MPN formation (%)
CONTROL	139	100^{a}	7.8 ± 0.3^{a}	61.9 ± 3.5^{a}	100
GSNO	128	93.0 ± 2.3^{a}	$6.5 \pm 0.4^{a,b}$	40.8 ± 2.6^{b}	100
L-NAME	136	1.5 ± 1^{c}	1.5 ± 0.5^{c}	13.8 ± 1.7^{c}	100
AG	146	57.5 ± 4.1^{b}	$2.5 \pm 0.2^{b,c}$	13.7 ± 1.3^{c}	100

n (number of evaluated oocytes per group); MPN (male pronucleus formation); $^{a, b, c}$ in the same column denote significant differences ($P<0.05$) between groups.

3.2.2. Experiment 2B: Effects of NO on the interaction between spermatozoa and decumulated oocytes

To verify that the effect of the inhibitors was not influenced by the presence of cumulus cells, we decided to evaluate the IVF outcome with denuded oocytes. The obtained results are shown in Table 4, in which we can observe that there was no penetration when the IVF medium was supplemented with L-NAME and was very low when using AG. The addition of the NO donor to the fertilization medium had no significant effect on the percentage of penetration with respect to the CONTROL group. As for the spermatozoa adhered to the ZP, both the NO donor and the NOS inhibitors lowered this parameter when compared to the CONTROL.

Table 4. Effects of NO during IVF with denuded oocytes.

Treatment	*n*	Penetration (%)	Sperm/Oocyte (n)	Sperm/ZP (%)	MPN formation (%)
CONTROL	61	98.4 ± 1.6^{a}	8.3 ± 0.5^{a}	41.2 ± 2.5^{a}	98.3 ± 0.2^{a}
GSNO	63	96.8 ± 2.2^{a}	8.0 ± 0.5^{a}	25.3 ± 1.3^{b}	98.4 ± 0.2^{a}
L-NAME	66	0	0	0.6 ± 0.1^{c}	0
AG	68	17.6 ± 4.6^{c}	1.8 ± 0.3^{b}	5 ± 0.9^{c}	100^{a}

n (number of evaluated oocytes per group); MPN (male pronucleus formation); $^{a, b, c}$ in the same column denote significant differences ($P<0.05$) between groups.

3.2.3. Experiment 2C: Effects of low NOS inhibitors concentration on the interaction between spermatozoa and decumulated oocytes

Furthermore, we decided to analyze if IVF results would be modified by decreasing the inhibitor concentration, as well as that of spermatozoa (which synthesize NO). We observed (Table 5) that the inhibitors continued to have the same effect on all analyzed parameters. Also, with a lower sperm concentration (2.5×10^4 spermatozoa/mL), the penetration percentage in the CONTROL and in the GSNO groups was lower than in our previous experiments, in which a 10-fold higher sperm concentration was used.

Table 5. Effects of NO during IVF with lower concentrations of sperm, NO donor and NOS inhibitors.

Treatment	*n*	Penetration (%)	Sperm/Oocyte (n)	Sperm/ZP (n)	MPN formation (%)
CONTROL	80	78.7 ± 4.6^a	5.5 ± 0.9	42.8 ± 4.6^a	96.8 ± 2.2
GSNO	76	77.6 ± 4.8^a	7.2 ± 0.9	36.4 ± 3.9^a	100
L-NAME	102	1.9 ± 1.4^b	1.0 ± 0	5.0 ± 0.6^b	100
AG	93	3.2 ± 1.8^b	1.0 ± 0	4.9 ± 0.8^b	100

n (number of evaluated oocytes per group); MPN (male pronucleus formation); $^{a, b}$ in the same column denote significant differences ($P<0.05$) between groups.

4. DISCUSSION

The participation of the NOS/NO system in the reproductive function has been widely demonstrated [34]. NO has a dual role. Low amounts, generated under physiological conditions, seem to be beneficial for sperm functions [7,28] but the excessive synthesis of NO, that takes place under in vitro fertilization conditions, could be detrimental for sperm function [47]. For that, the amount of NO in the fertilization media is variable and depends on the cumulus cells or sperm production, which could modify the capacitation process and the IVF outcome. The present study is, to the best of our knowledge, the first one to tackle both these aspects in porcine species, in the effort to obtain more insight on NO-mediated gamete interaction in vitro in this species.

NO synthesis takes place via L-Arginine oxidation by three distinct NOS isoforms: nNOS, eNOS and iNOS [48]. Numerous studies have been conducted to determine the presence and localization of these enzymes in sperm from several species [34] with slight differences between them [15,44,49]. However, the localization in boar sperm has not been described. We encountered a similar distribution between eNOS and nNOS, mostly in the sperm head region, whereas the immunofluorescent iNOS-staining was spread on almost all sperm regions. This pattern could have a physiological significance and it may suggest that the constitutive NOS could be closely related to the activation of key pathways

which leads to the capacitation [49], while the general distribution of the iNOS immunostaining might be connected to inflammatory processes in the male reproductive tract [50–52], rather than in the acquiring of the fertilization ability. We do not know if the NOS pattern exhibited by boar sperm changes during incubation *in vitro* but this aspect should be addressed in future studies.

In the porcine species, research was focused mainly to address the involvement of NO in the promotion of capacitation [16,53–55], lacking studies addressing the effect on sperm motility. In this sense, our results showed that despite no differences were found at the beginning of the incubation, medium supplementation with AG, which selectively inhibits iNOS [56], significantly reduced VSL and VAP at 30 min of incubation. These results are completely opposite from the ones reported by Alizadeh *et al*. in varicocelized rats [57], where AG was shown to improve sperm motility and mitochondrial membrane potential. But both results are not comparable as their experimental design included an AG injection daily for 10 weeks, while we treated ejaculated sperm for 30 minutes. Nevertheless, it is worth noting that the reduction in these parameters has been linked to low breeding performance in porcine species [58]. In relation to the lack of a visible effect of the GSNO supplementation on sperm motility, this result agrees with Zini *et al*. [46] in human sperm, who demonstrated that a low concentration of a NO-releasing agent (0.1 mM), equal to the one we used, had no effect on the percentage of sperm motility or of hyperactivation. In regard to L-NAME, we did not find any significant difference in our experiment, while the addition of 10 mM L-NAME was reported to inhibit bull sperm progressive motility [25]. However, as some studies describe, this inhibitor is more likely to exert its effect on the inhibition of sperm capacitation rather than affecting sperm motility.

The phosphorylation levels of PKA substrates and tyrosine are known to be indicative of sperm capacitation status [26] and evidence confirm that NO regulates both serine/threonine [59] and tyrosine phosphorylation [60]. Our data suggest that the use of GSNO as a NO donor had no significant effect on the total level of phospho-PKA substrates (i.e. serine and threonine phosphorylation) and phosphorylation of tyrosine residues. On the contrary, Herrero and colleagues [60] have suggested that NO-releasing molecules might accelerate the capacitation process. In fact, when using sodium nitroprusside (SNP) during human sperm capacitation, an increase of tyrosine phosphorylation was observed. Similarly, Thundathil *et al*. [59] reported that the NO generated by spermine NONOate lead to an increase in the phosphorylation levels of the threonine-glutamine-tyrosine motif in two different human sperm proteins. However, we observed a specific phosphorylation pattern for three PKA substrate species, ~75, ~55 and ~50 kDa, which showed a lower degree of phosphorylation in the presence of GSNO. These data also seem to be in contrast

with a previous work [61] and might be explained by the difference in the capacitation time (60 min in boar vs 240 min in human vs 90 min in mouse spermatozoa) and the species used, since the dynamics of serine/threonine phosphorylation are species-specific [62,63]. On the other hand, our results showed that the inhibition of NO synthesis lead to a decrease in the levels of phospho-PKA substrates. This effect was more evident in the ~75 and ~50 kDa species. We speculate that these bands might contain proteins targeted for tyrosine phosphorylation, after they have been phosphorylated in serine/threonine by PKA in the presence of NO [64] to allow the correct development of the capacitation process.

NO is able to determine an increase in Tyr-P via the sGC-cGMP signaling pathway [34] at nanomolar levels [65] and the lack of NO due to NOS inhibition is correlated with lower levels of Tyr-P [60,66]. However, according to our data, the NOS inhibitors had no effect on Tyr-P. It is possible that neither the NO donor nor the inhibitors used in our study were able to increase or lower Tyr-P because the low endogenous NO levels were enough to induce it [67]. This result is supported by our Tyr-P immunolocalization data, where no differences were observed between treatments.

At a molecular level, the AR shares a significant overlap with molecular events of capacitation [48] and both processes have been shown to be regulated by NO [46]. When incubating boar spermatozoa with exogenous NO, we did not observe any differences when compared to CONTROL. Other studies, however, report the NO donor's ability to increase the percentage of acrosome-reacted sperm in boars [54] and different species (human [68], buffalo [69] and mouse [61]). This discrepancy might be explained by the different NO-releasing molecule used in these studies, which have different kinetics for NO generation [60]. Interestingly, when adding L-NAME to the incubation medium, the AR was significantly reduced in our study. This finding is consistent with previous studies in boar [16,54], human [60] and hamster spermatozoa [70], which confirms that endogenous NO is necessary for spermatozoa to achieve their full fertilizing ability [60].

The translocation of PS is considered a physiological event during the capacitation process, but also a sign of cellular damage [20,38]. During sperm capacitation, the bicarbonate-stimulated protein phosphorylation pathway leads to the activation of phospholipid scramblase [37,71] which results in the exposure of PS at the outer membrane surface [37]. Our results showed that the use of GSNO did not induce apoptotic-like changes in sperm when compared to CONTROL. This contrasts the findings of Moran *et al*. [72] and the reason might be the different methodological approach, namely the use of a different NO-releasing compound and its concentration (100 μM GSNO vs 400 μM SNP). It has been reported that an increase in Annexin-positive spermatozoa is related to capacitation in boar semen [72] and that NOS inhibitors prevent capacitation [70]. This is in accordance with our

observations regarding the NOS inhibitor L-NAME, which lowered significantly the percentage of Annexin-positive sperm.

In sperm, $[Ca^{2+}]i$ changes through two routes, either Ca^{2+} ions are released from internal stores or transported into the cell by sperm-specific membrane channels [62,73]. Previous studies have shown that NO can interact with different Ca^{2+} routes [74–76] also in spermatozoa [27]. In this sense, we have investigated how the NOS/NO system regulates $[Ca^{2+}]i$ in porcine sperm. The results showed changes only when NOS inhibitors were used, L-NAME having the most potent effect. We hypothesized that in its presence, Ca^{2+} ions get expelled quickly from spermatozoa mainly through the Ca^{2+} efflux pump, Plasma Membrane Calcium ATPase 4 (PMCA4) [77,78], which is known to regulate NO signaling by down-regulating the NOS in murine sperm [77]. When using L-NAME, the PMCA4-NOS interaction might not have taken place, which in turn might have led PMCA4 to extrude the cytosolic Ca^{2+}. Further experiments are needed to test this hypothesis.

Although it also affects the rest of the NOS isoforms, AG preferentially inhibits the iNOS isoform [79], which could explain why the reduction of $[Ca^{2+}]i$ when adding this inhibitor is not as pronounced as it is with L-NAME. Our results suggest that in the beginning the Ca^{2+} output is compensated by the Ca^{2+} that comes from the internal stores causing the increase of $[Ca^{2+}]i$. Once the internal stores are empty, $[Ca^{2+}]i$ begins to decrease until reaching levels similar to those obtained with L-NAME. No significant differences were observed in relation to the GSNO supplementation, suggesting that NO contributes to the gradual increase of $[Ca^{2+}]i$. This effect may be observed as a consequence of NO-mediated S-nitrosylation on sperm Ca^{2+} stores such as the ryanodine receptors [27,80,81]. Clearly, more experiments will be needed to confirm these data.

We have shown that the inhibition of NO synthesis, mainly by L-NAME, affects protein phosphorylation, acrosome reaction and Ca^{2+} fluxes. However, the best test that indirectly evaluates sperm capacitation is the IVF [82], because only fully capacitated sperm can bind to the zona pellucida, undergo acrosome reaction and penetrate the oocyte's plasma membrane. Consequently, we studied the modulation of sperm capacitation by NO in an IVF system under three circumstances: i) IVF with cumulus-oocyte complexes, ii) IVF with denuded oocytes and iii) denuded oocytes with reduced concentrations of NO donor and inhibitors. The results showed that under these three circumstances the tendency was the same, that is, in the presence of NOS inhibitors, the number of spermatozoa adhered to the zona pellucida, the percentage of penetrated oocytes, and the mean number of spermatozoa per penetrated oocyte decreased. In addition, this effect was more pronounced when the L-NAME inhibitor was used.

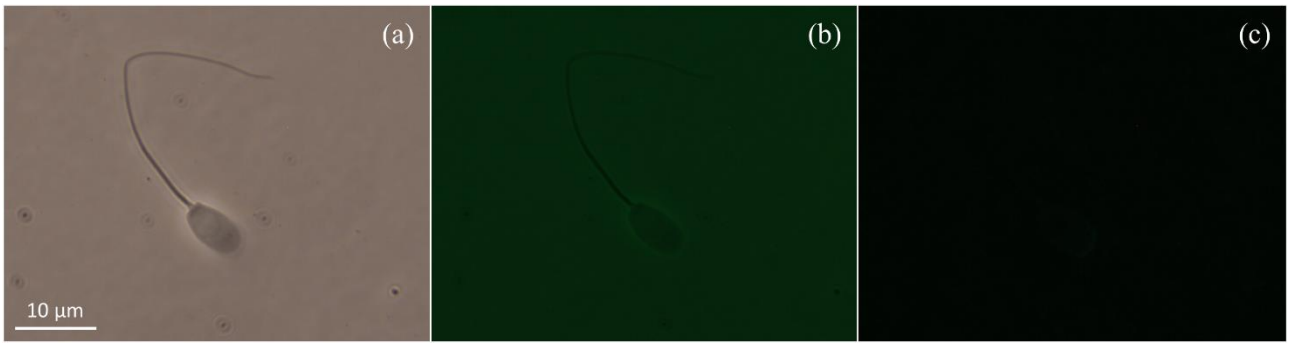
Although it was proved that spermatozoa can synthesize NO, the evidence that its synthesis is sufficient to be physiologically important is not very clear [83]. For this reason, the first part of our IVF experiments was done with oocytes together with cumulus cells which generate significant amounts of NO and, therefore, participate in the processes of capacitation and fertilization [27]. Under these circumstances, we observed that both NOS inhibitors (L-NAME and AG) decreased the penetration rate but in a different way: AG reduced this parameter to half versus CONTROL, while L-NAME reduced it almost to zero. This may lead us to believe that the inhibitory effect of AG on NO production from cumulus cells or spermatozoa is not total since AG is a less potent inhibitor of the constitutive isoforms [79]. For this reason, enough NO could still be produced by the constitutive isoforms thus allowing the capacitation in some spermatozoa.

In 2008, Hou *et al*. [54] observed that the addition of L-NAME inhibited NO production by 30–40%, impairing the ability of spermatozoa to undergo the acrosome reaction. However, in our experiment, the addition of L-NAME decreased the penetration rate almost to zero, the AR levels being also significantly reduced. Therefore, in these conditions we could assume that NO synthesis was almost completely abolished, which might be explained by the fact that the inhibitor concentration used in our study was higher than the one used by Hou and colleagues.

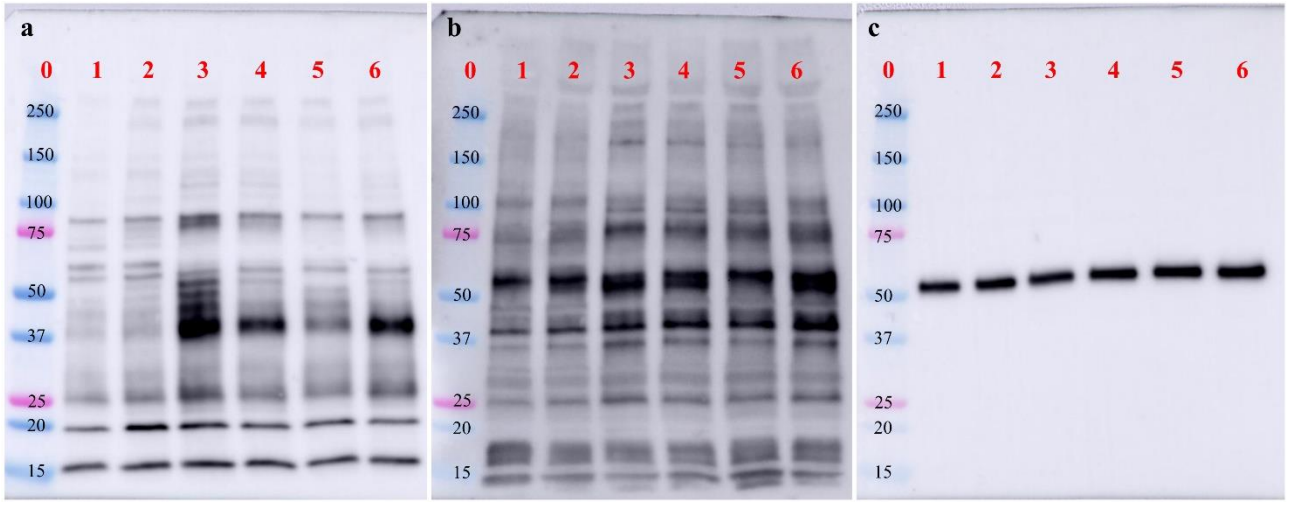
Since cumulus cells could be differently sensible to NOS inhibitors, we considered performing IVF using denuded oocytes. The results showed a big decrease in the penetration rate with the AG inhibitor and zero penetration with L-NAME, so in the first experiment, the cumulus cells even in the presence of inhibitors were able to generate NO to allow sperm capacitation and fertilization. Finally, with the purpose to check if the results previously obtained were due to the high concentration of NO donor, NOS inhibitors or high number of spermatozoa in the medium, we decided to reduce these parameters. We observed that the penetration in the CONTROL and GSNO groups decreased, but it didn't increase in the groups containing inhibitors. We can assume that NO sperm production continued being abolished. On the other hand, Leal *et al*. [25] obtained a penetration rate of 70% in bovine with the same L-NAME concentration. Perhaps in this species the constitutive NOS in sperm are less sensible to this inhibitor. In other species, such as human [10] or mouse [8], it has been shown that the inhibitor effects of L-NAME were dose-dependent and the oocyte penetration could be affected even without modifications in the sperm capacitation parameters [10]. In contrast to Francavilla *et al*. [10], who observed that constitutive NOS play a role in the human sperm's capacity to fuse with oocyte but not in the ZP binding, our results showed that even though the binding was not completely abolished, it decreased, so we can assume that the primary binding is less affected by NO absence. Interestingly, the NO donor GSNO lowered significantly the number of spermatozoa

bound to the ZP when compared with the CONTROL in either the presence or absence of cumulus cells. A similar finding was reported by Wu *et al*. [84], suggesting that physiologic levels of NO are required for the binding process.

5. SUPPORTING INFORMATION



Supplementary file for Figure 2. Negative control of the indirect immunofluorescence technique applied for NOSs localization. Spermatozoa were fixed, permeabilized, incubated with a FITC-labelled secondary antibody and examined under an epifluorescence microscope at x1000 magnification. Representative pictures are shown by phase-contrast microscopy (**a**), merging the phase-contrast image with the green fluorescence pattern (**b**) and for the immunofluorescent staining (**c**). No non-specific staining was observed.



Supplementary file for Figure 3. Representative membranes for phospho-PKA substrates (a), tyrosine phosphorylation (b) and β-tubulin (c). Red numbers indicate the content of each lane. (**0**) Precision Plus Protein™ Dual Color Standards (Bio-Rad, Madrid, Spain, #1610374). Molecular weights are expressed in kilodaltons. (**1-6**) Immunoblotted protein extracts from spermatozoa incubated under different experimental conditions, as follows: (**1**) Dulbecco's Phosphate-Buffered Saline without calcium chloride and magnesium chloride, time 0 h. (**2**) TALP medium, time 0 h. (**3**) CONTROL: TALP medium, time 1 h. (**4**) GSNO: TALP medium supplemented with 100 µM S-Nitrosoglutathione, time 1 h. (**5**) L-NAME: TALP medium

supplemented with 10 mM N^G-Nitro-L-Arginine Methyl Ester Hydrochloride, time 1 h. (**6**) AG: TALP medium supplemented with 10 mM Aminoguanidine Hemisulfate salt, time 1 h.

6. REFERENCES

[1] Aitken RJ. Reactive oxygen species as mediators of sperm capacitation and pathological damage. Mol Reprod Dev 2017;84:1039–52. doi:10.1002/mrd.22871.

[2] Wang J, He Q, Yan X, Cai Y, Chen J. Effect of exogenous nitric oxide on sperm motility in vitro. Biol Res 2014;47:44. doi:10.1186/0717-6287-47-44.

[3] Romero-Aguirregomezcorta J, Santa ÁP, García-Vázquez FA, Coy P, Matás C. Nitric oxide synthase (NOS) inhibition during porcine in vitro maturation modifies oocyte protein S-nitrosylation and in vitro fertilization. PLoS One 2014;9:e115044. doi:10.1371/journal.pone.0115044.

[4] Lefièvre L, Chen Y, Conner SJ, Scott JL, Publicover SJ, Ford WCL, et al. Human spermatozoa contain multiple targets for protein S-nitrosylation: An alternative mechanism of the modulation of sperm function by nitric oxide? Proteomics 2007;7:3066–84. doi:10.1002/pmic.200700254.

[5] Herrero MB, Viggiano JM, Pérez Martínez S, de Gimeno MF. Evidence that nitric oxide synthase is involved in progesterone-induced acrosomal exocytosis in mouse spermatozoa. Reprod Fertil Dev 1997;9:433–9.

[6] Gawronska B, Bodek G, Ziecik AJ. Distribution of NADPH-diaphorase and Nitric Oxide Synthase (NOS) in Different Regions of Porcine Oviduct During the Estrous Cycle. J Histochem Cytochem 2000;48:867–75. doi:10.1177/002215540004800615.

[7] Sengoku K, Tamate K, Yoshida T, Takaoka Y, Miyamoto T, Ishikawa M. Effects of low concentrations of nitric oxide on the zona pellucida binding ability of human spermatozoa. Fertil Steril 1998;69:522–7.

[8] Herrero MB, Viggiano JM, Pérez Martínez S, de Gimeno MF. Effects of nitric oxide synthase inhibitors on the outcome of in vitro fertilization in the mouse. Reprod Fertil Dev 1996;8:301–4.

[9] Reyes R, Vázquez MLS, Delgado NM. Detection and bioimaging of nitric oxide in bovine oocytes and sperm cells. Arch Androl 2004;50:303–9. doi:10.1080/01485010490448471.

[10] Francavilla F, Santucci R, Macerola B, Ruvolo G, Romano R. Nitric oxide synthase inhibition in human sperm affects sperm-oocyte fusion but not zona pellucida binding. Biol Reprod 2000;63:425–9.

[11] Doshi SB, Khullar K, Sharma RK, Agarwal A. Role of reactive nitrogen species in male infertility. Reprod Biol Endocrinol 2012;10:109. doi:10.1186/1477-7827-10-109.

[12] Gadea J, Matás C. Sperm factors related to in vitro penetration of porcine oocytes. Theriogenology 2000;54:1343–57.

[13] Petters RM, Wells KD. Culture of pig embryos. J Reprod Fertil Suppl 1993;48:61–73.

[14] Rath D, Long CR, Dobrinsky JR, Welch GR, Schreier LL, Johnson LA. In vitro production of sexed embryos for gender preselection: high-speed sorting of X-chromosome-bearing sperm to produce pigs after embryo transfer. J Anim Sci 1999;77:3346–52.

[15] Meiser H, Schulz R. Detection and localization of two constitutive NOS isoforms in bull spermatozoa. Anat Histol Embryol 2003;32:321–5.

[16] Aquila S, Giordano F, Guido C, Rago V, Carpino A. Nitric oxide involvement in the acrosome reaction triggered by leptin in pig sperm. Reprod Biol Endocrinol 2011;9:133. doi:10.1186/1477-7827-9-133.

[17] Matás C, Vieira L, García-Vázquez FA, Avilés-López K, López-Úbeda R, Carvajal JA, et al. Effects of centrifugation through three different discontinuous Percoll gradients on boar sperm function. Anim Reprod Sci 2011;127:62–72. doi:10.1016/j.anireprosci.2011.06.009.

[18] Navarrete FA, García-Vázquez FA, Alvau A, Escoffier J, Krapf D, Sánchez-Cárdenas C, et al. Biphasic role of calcium in mouse sperm capacitation signaling pathways. J Cell Physiol 2015;230:1758–69. doi:10.1002/jcp.24873.

[19] Kawano N, Araki N, Yoshida K, Hibino T, Ohnami N, Makino M, et al. Seminal vesicle protein SVS2 is required for sperm survival in the uterus. Proc Natl Acad Sci U S A 2014;111:4145–50. doi:10.1073/pnas.1320715111.

[20] Martí E, Pérez-Pé R, Colás C, Muiño-Blanco T, Cebrián-Pérez JA. Study of apoptosis-related markers in ram spermatozoa. Anim Reprod Sci 2008;106:113–32. doi:10.1016/j.anireprosci.2007.04.009.

[21] Tardif S, Dubé C, Bailey JL. Porcine sperm capacitation and tyrosine kinase activity are dependent on bicarbonate and calcium but protein tyrosine phosphorylation is only associated with calcium. Biol Reprod 2003;68:207–13.

[22] Matás C, Sansegundo M, Ruiz S, García-Vázquez FA, Gadea J, Romar R, et al. Sperm treatment affects capacitation parameters and penetration ability of ejaculated and epididymal boar spermatozoa. Theriogenology 2010;74:1327–40. doi:10.1016/j.theriogenology.2010.06.002.

[23] Grynkiewicz G, Poenie M, Tsien RY. A new generation of Ca2+ indicators with greatly improved fluorescence properties. J Biol Chem 1985;260:3440–50.

[24] Campbell G, Skillings JH. Nonparametric Stepwise Multiple Comparison Procedures. J Am Stat Assoc 1985;80:998–1003. doi:10.1080/01621459.1985.10478216.

[25] Leal ACMS, Caldas-Bussiere MC, Carvalho CSP de, Viana KS, Quirino CR. Role of nitric oxide on quality of freshly ejaculated bull spermatozoa during heparin-induced in vitro capacitation. Anim Reprod Sci 2009;116:38–49. doi:10.1016/j.anireprosci.2008.12.020.

[26] Soriano-Úbeda C, García-Vázquez FA, Romero-Aguirregomezcorta J, Matás C. Improving porcine in vitro fertilization output by simulating the oviductal environment. Sci Rep 2017;7:43616. doi:10.1038/srep43616.

[27] Machado-Oliveira G, Lefievre L, Ford C, Herrero MB, Barratt C, Connolly TJ, et al. Mobilisation of Ca2+ stores and flagellar regulation in human sperm by S-nitrosylation: a role for NO synthesised in the female reproductive tract. Development 2008;135:3677–86. doi:10.1242/dev.024521.

[28] Donnelly ET, Lewis SE, Thompson W, Chakravarthy U. Sperm nitric oxide and motility: the effects of nitric oxide synthase stimulation and inhibition. Mol Hum Reprod 1997;3:755–62.

[29] Lewis SE, Donnelly ET, Sterling ES, Kennedy MS, Thompson W, Chakravarthy U. Nitric oxide synthase and nitrite production in human spermatozoa: evidence that endogenous nitric oxide is beneficial to sperm motility. Mol Hum Reprod 1996;2:873–8.

[30] Rosselli M, Dubey RK, Imthurn B, Macas E, Keller PJ. Effects of nitric oxide on human spermatozoa: evidence that nitric oxide decreases sperm motility and induces sperm toxicity. Hum Reprod 1995;10:1786–90.

[31] Weinberg JB, Doty E, Bonaventura J, Haney AF. Nitric oxide inhibition of human sperm motility. Fertil Steril 1995;64:408–13.

[32] Battistone MA, Da Ros VG, Salicioni AM, Navarrete FA, Krapf D, Visconti PE, et al. Functional human sperm capacitation requires both bicarbonate-dependent PKA activation and down-regulation of Ser/Thr phosphatases by Src family kinases. Mol Hum Reprod 2013;19:570–80. doi:10.1093/molehr/gat033.

[33] Belén Herrero M, Chatterjee S, Lefièvre L, De Lamirande E, Gagnon C. Nitric oxide interacts with the cAMP pathway to modulate capacitation of human spermatozoa. Free Radic Biol Med 2000;29:522–36. doi:10.1016/S0891-5849(00)00339-7.

[34] Staicu F-D, Matas Parra C. Nitric Oxide: Key Features in Spermatozoa. In: Nitric Oxide Synthase - Simple Enzyme-Complex Roles, Seyed Soheil Saeedi Saravi, InTechOpen; 2017, p. 137–54. doi:10.5772/67551.

[35] Töpfer-Petersen E. Molecules on the sperm's route to fertilization. J Exp Zool 1999;285:259–66.

[36] de Lamirande E, Leclerc P, Gagnon C. Capacitation as a regulatory event that primes spermatozoa for the acrosome reaction and fertilization. Mol Hum Reprod 1997;3:175–94.

[37] Gadella BM, Harrison RAP. Capacitation induces cyclic adenosine 3',5'-monophosphate-dependent, but apoptosis-unrelated, exposure of aminophospholipids at the apical head plasma membrane of boar sperm cells. Biol Reprod 2002;67:340–50.

[38] Aitken RJ, Baker MA, Nixon B. Are sperm capacitation and apoptosis the opposite ends of a continuum driven by oxidative stress? Asian J Androl 2015;17:633–9. doi:10.4103/1008-682X.153850.

[39] Abou-haila A, Tulsiani DRP. Signal transduction pathways that regulate sperm capacitation and the acrosome reaction. Arch Biochem Biophys 2009;485:72–81. doi:10.1016/j.abb.2009.02.003.

[40] Rosselli M, Dubey RK, Rosselli MA, Macas E, Fink D, Lauper U, et al. Identification of nitric oxide synthase in human and bovine oviduct. Mol Hum Reprod 1996;2:607–12.

[41] Lapointe J, Roy M, St-Pierre I, Kimmins S, Gauvreau D, MacLaren LA, et al. Hormonal and spatial regulation of nitric oxide synthases (NOS) (neuronal NOS, inducible NOS, and endothelial NOS) in the oviducts. Endocrinology 2006;147:5600–10. doi:10.1210/en.2005-1548.

[42] Tao Y, Fu Z, Zhang M, Xia G, Yang J, Xie H. Immunohistochemical localization of inducible and endothelial nitric oxide synthase in porcine ovaries and effects of NO on antrum formation and oocyte meiotic maturation. Mol Cell Endocrinol 2004;222:93–103. doi:10.1016/j.mce.2004.04.014.

[43] Herrero MB, Goin JC, Boquet M, Canteros MG, Franchi AM, Perez Martinez S, et al. The nitric oxide synthase of mouse spermatozoa. FEBS Lett 1997;411:39–42.

[44] O'Bryan MK, Zini A, Cheng CY, Schlegel PN. Human sperm endothelial nitric oxide synthase expression: correlation with sperm motility. Fertil Steril 1998;70:1143–7. doi:10.1016/S0015-0282(98)00382-3.

[45] O'Flaherty C, de Lamirande E, Gagnon C. Positive role of reactive oxygen species in mammalian sperm capacitation: triggering and modulation of phosphorylation events. Free Radic Biol Med 2006;41:528–40. doi:10.1016/j.freeradbiomed.2006.04.027.

[46] Zini A, De Lamirande E, Gagnon C. Low levels of nitric oxide promote human sperm capacitation in vitro. J Androl 1995;16:424–31.

[47] O'Flaherty C, Rodriguez P, Srivastava S. L-arginine promotes capacitation and acrosome reaction in cryopreserved bovine spermatozoa. Biochim Biophys Acta 2004;1674:215–21. doi:10.1016/j.bbagen.2004.06.020.

[48] Buzadzic B, Vucetic M, Jankovic A, Stancic A, Korac A, Korac B, et al. New insights into male (in)fertility: the importance of NO. Br J Pharmacol 2015;172:1455–67. doi:10.1111/bph.12675.

[49] Herrero MB, Pérez Martínez S, Viggiano JM, Polak JM, de Gimeno MF. Localization by indirect immunofluorescence of nitric oxide synthase in mouse and human spermatozoa. Reprod Fertil Dev 1996;8:931–4.

[50] Kushwaha S, Jena GB. Telmisartan ameliorates germ cell toxicity in the STZ-induced diabetic rat: studies on possible molecular mechanisms. Mutat Res 2013;755:11–23. doi:10.1016/j.mrgentox.2013.04.013.

[51] Patel S, Santani D. Role of NF-kappa B in the pathogenesis of diabetes and its associated complications. Pharmacol Rep 2009;61:595–603.

[52] Heeba GH, Hamza AA. Rosuvastatin ameliorates diabetes-induced reproductive damage via suppression of oxidative stress, inflammatory and apoptotic pathways in male rats. Life Sci 2015;141:13–9. doi:10.1016/j.lfs.2015.09.015.

[53] Funahashi H. Induction of capacitation and the acrosome reaction of boar spermatozoa by L-arginine and nitric oxide synthesis associated with the anion transport system. Reproduction 2002;124:857–64.

[54] Hou M-L, Huang S-Y, Lai Y-K, Lee W-C. Geldanamycin augments nitric oxide production and promotes capacitation in boar spermatozoa. Anim Reprod Sci 2008;104:56–68. doi:10.1016/j.anireprosci.2007.01.006.

[55] Aquila S, Giordano F, Guido C, Rago V, Carpino A. Insulin affects sperm capacity in pig through nitric oxide. Asian J Androl 2013;15:835–7. doi:10.1038/aja.2012.168.

[56] Corbett JA, Tilton RG, Chang K, Hasan KS, Ido Y, Wang JL, et al. Aminoguanidine, a novel inhibitor of nitric oxide formation, prevents diabetic vascular dysfunction. Diabetes 1992;41:552–6.

[57] Alizadeh R, Navid S, Abbasi N, Yari A, Mazaheri Z, Daneshi E, et al. The effect of aminoguanidine on sperm motility and mitochondrial membrane potential in varicocelized rats. Iran J Basic Med Sci 2016;19:1279–84. doi:10.22038/ijbms.2016.7908.

[58] Lee WY, Lee R, Kim HC, Lee KH, Cui XS, Kim NH, et al. Pig Spermatozoa Defect in Acrosome Formation Caused Poor Motion Parameters and Fertilization Failure through Artificial Insemination and In vitro Fertilization. Asian-Australasian J Anim Sci 2014;27:1417–25. doi:10.5713/ajas.2014.14206.

[59] Thundathil J, de Lamirande E, Gagnon C. Nitric oxide regulates the phosphorylation of the threonine-glutamine-tyrosine motif in proteins of human spermatozoa during capacitation. Biol Reprod 2003;68:1291–8. doi:10.1095/biolreprod.102.008276.

[60] Herrero MB, de Lamirande E, Gagnon C. Nitric oxide regulates human sperm capacitation and protein-tyrosine phosphorylation in vitro. Biol Reprod 1999;61:575–81. doi:10.1095/biolreprod61.3.575.

[61] Rahman MS, Kwon W-S, Lee J-S, Kim J, Yoon S-J, Park Y-J, et al. Sodium nitroprusside suppresses male fertility in vitro. Andrology 2014;2:899–909. doi:10.1111/j.2047-2927.2014.00273.x.

[62] Jin S-K, Yang W-X. Factors and pathways involved in capacitation: how are they regulated? Oncotarget 2017;8:3600–27. doi:10.18632/oncotarget.12274.

[63] Naresh S, Atreja SK. Detection, Localization and Tyrosine Phosphorylation Status of Ser/Thr Protein Phosphatase1γ in Freshly Ejaculated, In Vitro Capacitated and Cryopreserved Buffalo Spermatozoa. Reprod Domest Anim 2015;50:901–9. doi:10.1111/rda.12598.

[64] Visconti PE, Westbrook VA, Chertihin O, Demarco I, Sleight S, Diekman AB. Novel signaling pathways involved in sperm acquisition of fertilizing capacity. J Reprod Immunol 2002;53:133–50.

[65] Martínez-Ruiz A, Cadenas S, Lamas S. Nitric oxide signaling: classical, less classical, and nonclassical mechanisms. Free Radic Biol Med 2011;51:17–29. doi:10.1016/j.freeradbiomed.2011.04.010.

[66] Roy SC, Atreja SK. Tyrosine phosphorylation of a 38-kDa capacitation-associated buffalo (Bubalus bubalis) sperm protein is induced by L-arginine and regulated through a cAMP/PKA-independent pathway. Int J Androl 2008;31:12–24. doi:10.1111/j.1365-2605.2007.00745.x.

[67] de Andrade AFC, Arruda RP, Torres MA, Pieri NCG, Leite TG, Celeghini ECC, et al. Nitric oxide in frozen-thawed equine sperm: Effects on motility, membrane integrity and sperm capacitation. Anim Reprod Sci 2018;195:176–84. doi:10.1016/j.anireprosci.2018.05.022.

[68] Revelli A, Soldati G, Costamagna C, Pellerey O, Aldieri E, Massobrio M, et al. Follicular fluid proteins stimulate nitric oxide (NO) synthesis in human sperm: a possible role for NO in acrosomal reaction. J Cell Physiol 1999;178:85–92. doi:10.1002/(SICI)1097-4652(199901)178:1<85::AID-JCP11>3.0.CO;2-Y.

[69] Siddique RA, Atreja SK. Effect of Spermine-NONOate on acrosome reaction and associated protein tyrosine phosphorylation in Murrah buffalo (Bubalus bubalis) spermatozoa. Anim Reprod Sci 2012;131:81–7. doi:10.1016/j.anireprosci.2012.02.010.

[70] Kameshwari DB, Siva AB, Shivaji S. Inhibition of in vitro capacitation of hamster spermatozoa by nitric oxide synthase inhibitors. Cell Mol Biol (Noisy-Le-Grand) 2003;49:421–8.

[71] Bevers EM, Comfurius P, Dekkers DW, Zwaal RF. Lipid translocation across the plasma membrane of mammalian cells. Biochim Biophys Acta 1999;1439:317–30.

[72] Moran JM, Madejón L, Ortega Ferrusola C, Peña FJ. Nitric oxide induces caspase activity in boar spermatozoa. Theriogenology 2008;70:91–6. doi:10.1016/j.theriogenology.2008.02.010.

[73] Visconti PE. Understanding the molecular basis of sperm capacitation through kinase design. Proc Natl Acad Sci U S A 2009;106:667–8. doi:10.1073/pnas.0811895106.

[74] Trepakova ES, Cohen RA, Bolotina VM. Nitric oxide inhibits capacitative cation influx in human platelets by promoting sarcoplasmic/endoplasmic reticulum Ca2+-ATPase-dependent refilling of Ca2+ stores. Circ Res 1999;84:201–9.

[75] Ji J, Benishin CG, Pang PK. Nitric oxide selectively inhibits intracellular Ca++ release elicited by inositol trisphosphate but not caffeine in rat vascular smooth muscle. J Pharmacol Exp Ther 1998;285:16–21.

[76] Blatter LA, Wier WG. Nitric oxide decreases [Ca2+]i in vascular smooth muscle by inhibition of the calcium current. Cell Calcium 1994;15:122–31.

[77] Olli KE, Li K, Galileo DS, Martin-DeLeon PA. Plasma membrane calcium ATPase 4 (PMCA4) co-ordinates calcium and nitric oxide signaling in regulating murine sperm functional activity. J Cell Physiol 2018;233:11–22. doi:10.1002/jcp.25882.

[78] Cartwright EJ, Oceandy D, Neyses L. Physiological implications of the interaction between the plasma membrane calcium pump and nNOS. Pflugers Arch 2009;457:665–71. doi:10.1007/s00424-008-0455-z.

[79] Misko TP, Moore WM, Kasten TP, Nickols GA, Corbett JA, Tilton RG, et al. Selective inhibition of the inducible nitric oxide synthase by aminoguanidine. Eur J Pharmacol 1993;233:119–25.

[80] Kakizawa S. Nitric Oxide-Induced Calcium Release: Activation of Type 1 Ryanodine Receptor, a Calcium Release Channel, through Non-Enzymatic Post-Translational Modification by Nitric Oxide. Front Endocrinol (Lausanne) 2013;4:142. doi:10.3389/fendo.2013.00142.

[81] Hart JD, Dulhunty AF. Nitric oxide activates or inhibits skeletal muscle ryanodine receptors depending on its concentration, membrane potential and ligand binding. J Membr Biol 2000;173:227–36.

[82] Brackett BG, Bousquet D, Boice ML, Donawick WJ, Evans JF, Dressel MA. Normal development following in vitro fertilization in the cow. Biol Reprod 1982;27:147–58.

[83] Herrero MB, Gagnon C. Nitric oxide: a novel mediator of sperm function. J Androl 2001;22:349–56.

[84] Wu T-P, Huang B-M, Tsai H-C, Lui M-C, Liu M-Y. Effects of nitric oxide on human spermatozoa activity, fertilization and mouse embryonic development. Arch Androl 2004;50:173–9. doi:10.1080/01485010490425494.

CHAPTER 2

Nitric oxide-targeted protein phosphorylation during human sperm capacitation

ABSTRACT

Among many other molecules, NO insures the correct progress of sperm capacitation by mediating phosphorylation events. For a more comprehensive understanding of how this happens, we capacitated human spermatozoa from healthy men in the presence/absence of GSNO, a NO donor, two NOS inhibitors, L-NAME and AG and, finally, with/without L-Arginine, the substrate for NO synthesis, and/or FF. When analyzing the phosphorylation of PKA substrates and tyrosine residues, we particularly observed how the inhibition of NO synthesis affects certain protein bands (~ 110, ~ 87, ~ 75 and ~ 62 kDa) by lowering significantly their phosphorylation degree ($P<0.05$), even when spermatozoa were incubated with L-Arginine and/or FF. Mass spectrometry analysis identified 29 proteins in these species, which are related to: spermatogenesis, binding to the zona pellucida, energy and metabolism, stress response, motility and structural organization, signaling and protein turnover. These findings provide a deeper understanding of NO's role in the capacitation process, and consequently, future studies in infertile patients should determine how NO mediates phosphorylation events in the species here described.

1. INTRODUCTION

NO is a free radical which can regulate several physiological and pathological processes in mammals [1]. The cellular rates of NO synthesis mainly depend on NOS's activity and the availability of their substrate (L-Arginine) and co-factors [2]. The presence and localization of these enzymes have been extensively studied in spermatozoa belonging to different species [3], including humans [4,5] although their effect on sperm capacitation has not yet been fully understood.

Consequently, various studies hypothesized and investigated NO's involvement in the acquisition of sperm fertilizing ability. Most of the evidence supports the view that at levels exceeding physiologic concentrations, disruption of sperm function occurs, but that at low levels, NO is essential for sperm function [6]. It has been shown that, at physiologic levels, NO plays an important role in sperm capacitation [7,8], acrosome reaction [9] and in the maintenance of sperm motility [10].

The development and completion of the capacitation process require post-translational modifications of different proteins, particularly phosphorylation events, the dynamics of which are species-specific [11]. NO can modulate the capacitation via protein S-nitrosylation [12] and activation of the cAMP/PKA pathway [13]. The latter leads to an increase in the phosphorylation levels of sperm proteins, particularly in serine, threonine and tyrosine residues [14,15]. Moreover, it has been suggested that the S-nitrosylation of AC may be a possible mechanism of action of NO [16].

Another pathway through which NO can modulate protein phosphorylation is by activating the sGC and, thus, increasing the intracellular concentration of cGMP [17]. cGMP activates PKG [18,19] which leads to the serine/threonine phosphorylation of proteins that promote sperm capacitation and acrosome reaction [20,21]. Interestingly, since cGMP and cAMP compete for the catalytic sites of phosphodiesterases [22,23], an increase in the intracytoplasmic cGMP concentration may inhibit cAMP degradation via cyclic nucleotide phosphodiesterase type 3 [24]. The higher cAMP levels can, then, activate PKA and protein tyrosine phosphorylation.

The ability to synthesize NO has not been described only in spermatozoa, but in oocytes and cumulus cells as well [3]. In fact, NO's presence has been detected in the FF after gonadotropin stimulation [25]. Additionally, Revelli *et al*. [9] demonstrated that a protein-enriched FF solution can increase the endogenous NOS activity in human sperm, thus leading to acrosome reaction in the same cells.

For these reasons, the aim of this study was to further investigate NO's involvement in PKA activation and phosphorylation of tyrosine residues during the in vitro capacitation of human spermatozoa and how these phosphorylation events are regulated by the presence of FF.

2. MATERIALS AND METHODS

Ethics

The procedures carried out in this study were approved by the Ethics Review Committee of CEIC Hospital General Universitario Jose Maria Morales Meseguer (Murcia, Spain) (Approval No. EST: 06/17). Men and women participating in the gamete donation program at IVI-RMA Global (Murcia, Spain) were invited to participate in the study. All the donors who accepted provided their signed informed consent.

Materials

Unless otherwise stated, chemicals and reagents were purchased from Sigma-Aldrich Quimica S.A. (Madrid, Spain). Sydney IVF Sperm Medium was provided by Cook Medical (Barcelona, Spain). 4-15% polyacrylamide gels were supplied by Bio-Rad (Madrid, Spain) and PVDF membranes by Merck (Madrid, Spain). The primary antibodies were: anti-phospho-PKA substrates (PKAs-P) (Cell Signaling Technology, Beverly, USA, #9624), anti-phosphotyrosine (Tyr-P) (Abcam, Cambridge, UK, #ab10321) and anti-β-tubulin (β-TUB) (Sigma-Aldrich Quimica S.A., Madrid, Spain, #T0198). Horseradish peroxidase-conjugated anti-rabbit and anti-mouse IgGs were obtained from Santa Cruz Biotechnology (Heidelberg, Germany, #sc2004) and Bio-Rad (Madrid, Spain, #1706516), respectively. PageBlue was provided by Thermo Scientific (Rockford, IL, USA), whereas acetonitrile, trifluoroacetic acid and formic acid by Fisher Scientific (UK). Trypsin Gold Proteomics Grade and ProteaseMax surfactant were purchased from Promega Corporation (Madison, MI, USA).

Sperm samples

Semen samples were obtained by masturbation after 3-5 days of sexual abstinence from 7 donors. All samples were allowed to liquefy for at least 30 min at 37 °C; then they were evaluated for sperm concentration, motility, and morphology according to the World Health Organization guidelines (2010). Only specimens with normal parameters were used in the experiments.

FF samples

FF samples were obtained from 26 women taking part in the oocyte donation program at IVI-RMA Global (Murcia, Spain). Ovarian stimulation was achieved by administering a human recombinant follicle-stimulating hormone and a gonadotrophin-releasing hormone antagonist, as previously described [26]. When the follicles reached an average diameter of 17.5-18 mm, the ovulation was triggered with a GnRH agonist [26]. Approximately thirty-six hours later, dominant

follicles were punctured transvaginally under ultrasound guidance, and FF was aspirated together with the oocyte. After oocyte retrieval, only visibly blood-free FF samples (n = 27) were further processed. FF was centrifuged during 15 min at 1500 × g. The supernatant was filtered using 0.22 μm filter units (Merck KGaA, Darmstadt, Germany) to remove cellular debris, then aliquoted and stored at -20 °C until use. Before and after centrifugation, an aliquot from each FF sample was used to determine hemoglobin (Hb) levels with a HemoCue Plasma/Low Hb System (Ängelholm, Sweden) to ensure that, after being processed, the FF did not contain detectable levels of Hb, which is a NO scavenger [27].

SDS-PAGE and WB

Sperm protein extracts were obtained, separated by electrophoresis and immunoblotted following a method adapted from Soriano-Úbeda *et al*. [28]. Briefly, spermatozoa were collected by centrifugation, washed in 600 μL of phosphate buffer solution without calcium chloride and magnesium chloride (PBS), resuspended in Laemmli sample buffer [29], boiled for 5 min and centrifuged once more. Supernatants were then supplemented with 5% *v/v* β-mercaptoethanol and boiled again for 3 min. Next, the protein extracts equivalent to 1×10^6 sperm were loaded per lane, subjected to SDS-PAGE at 80 mA and electro-transferred to PVDF membranes at 250 mA for 75 min on ice. For PKAs-P and β-TUB immunodetections, membranes were blocked for 1 h at room temperature with 5% *w/v* BSA in TBS containing 0.1% *v/v* Tween 20 (TTBS), whereas for Tyr-P a 5% *w/v* BSA in PBS containing 0.1% *v/v* Tween 20 (TPBS) was used for the same time. Incubations with the primary antibodies were performed as follows: 1:2,000 in 5% *w/v* BSA-TTBS (overnight at 4 °C) for PKAs-P; 1:10,000 in 1% *w/v* BSA-TPBS (2 h at room temperature) for Tyr-P; 1:5,000 in 1% *w/v* BSA-TTBS for β-TUB (overnight at 4 °C). As far as the secondary antibodies are concerned, the incubations were done as follows: anti-rabbit, 1:10,000 in 5% *w/v* BSA-TTBS (2 h at room temperature) for PKAs-P; anti-mouse, 1:10,000 in 1% *w/v* BSA-TPBS (1 h at room temperature) for Tyr-P; 1:10,000 in 1% *w/v* BSA-TTBS (1 h at room temperature) for β-TUB. After each antibody incubation, the membranes were washed 3 × 5 min with either TTBS or TPBS. When necessary, PVDF membranes were stripped at 60 °C for 20 min in 2% *w/v* SDS, 0.74% *v/v* β-mercaptoethanol, 62.5 mM Tris, pH 6.5, and washed 5 × 5 min in TTBS. Blots were visualized by chemiluminescence (Amersham Imager 600, GE Healthcare Life Sciences, Buckinghamshire, UK) using a Pierce® ECL 2 Western Blotting Substrate (80196, Lumigen Inc, Southfield, MI, USA) according to the manufacturer's instructions. The relative amount of signal in each membrane was quantified using the ImageQuant TL v8.1 software (GE Healthcare). β-TUB was used as a loading control and the signal

for each lane was normalized to its corresponding β-TUB value. Molecular masses were expressed in kilodaltons (kDa).

SDS-PAGE gel staining and in-gel trypsin digestion

Once the immunoblots were analyzed, a sample of sperm protein extracts was subjected to electrophoresis, as described in the previous section, followed by gel staining. Briefly, the gel was washed 3 × 10 min with Milli-Q water and stained overnight at room temperature with PageBlue. Next, to destain the gel, it was rinsed twice and then washed for 4 × 15 min with Milli-Q water.

The selected protein bands were spliced in approximately 2 × 2 mm parts, then they were washed twice with Milli-Q water and then twice with 25 mM ammonium bicarbonate buffer pH 8.5 in 50% *v/v* acetonitrile during 30 min at 37 °C. After removing the supernatant, bands were dried for 15 min using an Eppendorf 5301 vacuum evaporator, and then they were incubated with 100 µL of 25 mM ammonium bicarbonate buffer pH 8.5 with 20 mM DTT at 56 °C for 20 min. The supernatant was removed and the samples were alkylated by adding 100 µL of 25 mM ammonium bicarbonate buffer pH 8.5 with 100 mM iodoacetamide during 30 min at room temperature in the dark. The supernatant was again removed and the gel spots were washed first with 25 mM ammonium bicarbonate buffer pH 8.5 and then with 25 mM ammonium bicarbonate buffer pH 8.5 in 50% *v/v* acetonitrile during 15 min at 37 °C each time. After washing, the gel spots were dried again and then incubated with 50 µL 25 mM ammonium bicarbonate buffer pH 8.5 containing 0.5 µg of Trypsin Gold Proteomics Grade and 0.01% *w/v* ProteaseMax surfactant during 10 min at 4 °C. Next, the samples were submitted to digestion during at least 3 h at 37 °C. The supernatant was collected in a new tube and evaporated. To enhance the extraction of digested fragments from the remaining gel spots, they were washed with 100 µL of a solution containing 50% *v/v* acetonitrile and 0.5% *v/v* trifluoroacetic acid and then with 100 µL of acetonitrile during 30 min at 37 °C each time. Finally, after these washing steps, both supernatants were collected in the same tube and dried using the vacuum evaporator.

HPLC-ESI-Q-TOF-MS/MS analysis

The separation and analysis of the tryptic digests of the samples were performed with a HPLC/MS system consisting of an Agilent 1290 Infinity II Series HPLC (Agilent Technologies, Santa Clara, CA, USA) equipped with an Automated Multisampler Module and a High-Speed Binary Pump, connected to an Agilent 6550 Q-TOF Mass Spectrometer (Agilent Technologies, Santa Clara, CA, USA) using an Agilent Jet Stream Dual electrospray (AJS-Dual ESI) interface. Experimental parameters for HPLC and Q-TOF were set in the MassHunter Workstation Data Acquisition software (Agilent Technologies, Rev. B.08.00).

Dry samples from trypsin digestion were resuspended in 20 µL of buffer A, consisting in water/acetonitrile/formic acid (94.9:5:0.1). The samples were injected onto an Agilent AdvanceBio Peptide Mapping HPLC column (2.7 µm, 100 × 2.1 mm, Agilent Technologies), thermostated at 55 °C, at a flow rate of 0.4 mL/min. After the injection, the column was washed with buffer A for 2 min and the digested peptides were eluted using a linear gradient 0-40% with buffer B (water/acetonitrile/formic acid, 10:89.9:0.1) for 30 min.

The mass spectrometer was operated in the positive mode. The nebulizer gas pressure was set to 35 psi, whereas the drying gas flow was set to 14 L/min at a temperature of 300 °C, and the sheath gas flow was set to 11 L/min at a temperature of 250 ºC. The capillary spray, fragmentor and octopole RF Vpp voltages were 3500 V, 360 V and 750 V, respectively. Profile data were acquired for both MS and MS/MS scans in extended dynamic range mode. MS and MS/MS mass range were 50-1700 m/z and scan rates were 8 spectra/sec for MS and 3 spectra/sec for MS/MS. Auto MS/MS mode was used with precursor selection by abundance and a maximum of 20 precursors were selected per cycle. A ramped collision energy was used with a slope of 3.6 and an offset of -4.8. The same ion was rejected after two consecutive scans.

Data processing and analysis was performed with the Spectrum Mill MS Proteomics Workbench software (Rev B.06.00.201, Agilent Technologies, Santa Clara, CA, USA). Briefly, raw data were extracted under default conditions as follows: unmodified or carbamidomethylated cysteines; [MH]+ 50–10000 m/z; maximum precursor charge +5; minimum signal-to-noise MS (S/N) 25; finding ^{12}C signals.

The MS/MS search against the appropriate and updated protein database was performed with the following criteria: variable modifications search mode (carbamidomethylated cysteines, STY phosphorylation, oxidized methionine, and N-terminal glutamine conversion to pyroglutamic acid); tryptic digestion with 5 maximum missed cleavages; ESI-Q-TOF instrument; minimum matched peak intensity 50%; maximum ambiguous precursor charge +5; monoisotopic masses; peptide precursor mass tolerance 20 ppm; product ion mass tolerance 50 ppm; and calculation of reversed database scores. Validation of peptide and protein data was performed using auto thresholds. General confidence criteria for peptide validation were: score > 9; SPI > 70%.

Statistical analysis

The data are presented as the mean ± standard error of the mean (SEM). Differences between mean values of multiple groups were analyzed by One-Way ANOVA followed by Tukey test. The

level of significance was set at $P<0.05$. All statistical analyses were conducted using IBM SPSS Statistics for Windows, Version 20.0 (IBM, Armonk, NY, USA).

Experimental design

To investigate how NO modulates the phosphorylation of PKA substrates and tyrosine residues, sperm samples were capacitated in Sydney IVF Sperm Medium for 4 h at 37 °C and 6% CO_2 with different treatments (Fig. 1). The following experimental groups were established depending on the treatment used: CONTROL: spermatozoa incubated in the absence of any treatment; GSNO: spermatozoa incubated in the presence of 100 µM S-Nitrosoglutathione, a NO donor; L-NAME: spermatozoa incubated in the presence of 10 mM N^G-Nitro-L-Arginine Methyl Ester Hydrochloride, a NOS inhibitor; AG: spermatozoa incubated in the presence of 10 mM Aminoguanidine Hemisulfate salt, another NOS inhibitor.

To determine whether the addition of L-Arginine, the substrate for NO synthesis, and FF has an effect on phospho-PKA substrates and Tyr-P, the capacitation medium was supplemented or not with 10 mM L-Arginine and/or 20% *v/v* FF (Fig. 1). These concentrations were chosen based on a literature review [12,30–33].

The experimental groups mentioned above were subjected to WB analysis to detect differences in the levels of phospho-PKA substrates and Tyr-P. The protein bands that showed significant differences amongst the treatments mentioned above were isolated and subjected to in-gel trypsin digestion, followed by mass spectrometry analysis, to identify their proteomic profile.

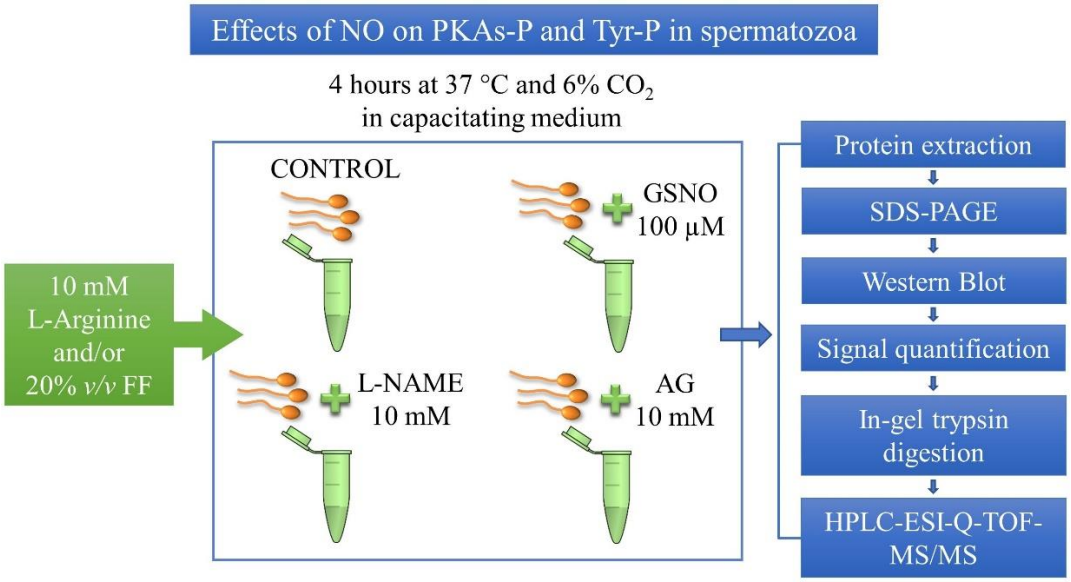


Figure 1. Experimental design. Human spermatozoa were capacitated for 4 hours in the presence/absence of a NO donor (GSNO) and two NOS inhibitors (L-NAME and AG). The experimental groups were supplemented or not with L-Arginine and/or follicular fluid (FF). Sperm proteins were subjected to

electrophoresis followed by Western Blot to analyze the phosphorylation levels of phospho-PKA substrates (PKAs-P) and tyrosine residues (Tyr-P). The amount of signal in each membrane was determined by chemiluminescence and, subsequently, quantified. Specific protein bands, that showed significant differences amongst the treatments mentioned above, were subjected to in-gel trypsin digestion, followed by mass spectrometry analysis (HPLC-ESI-Q-TOF-MS/MS).

3. RESULTS

Phospho-PKA substrates and Tyr-P

Many studies evidenced that for spermatozoa to achieve their fertilizing ability, a fine regulation of protein phosphorylation is required, which in part depends on NO (summarized by [34]). Total levels of phospho-PKA substrates were significantly lowered by L-NAME and AG, when compared to the NO donor ($P<0.05$; Fig. 2a and d). The NO donor significantly increased the phosphorylation degree of two PKA substrates of approximately ~ 87 kDa and ~ 62 kDa, when compared to CONTROL (Fig. 2a and e). The presence of NOS inhibitors in the capacitation medium lowered significantly the phosphorylation degree of the ~ 87 kDa species, compared to the CONTROL and GSNO groups, and of the ~ 62 kDa species, only when compared to GSNO (Fig. 2a and e). When adding L-Arginine, no differences were observed in the overall levels of phospho-PKA substrates (Fig. 3a and d). However, the relative amount of signal for the ~ 87 kDa species was lower with the L-NAME inhibitor than in the GSNO group ($P<0.05$; Fig. 3a and e). Supplementation of the sperm medium with FF did not affect significantly the global phosphorylation of PKA substrates when compared to CONTROL (Fig. 4a and d), but it decreased the phosphorylation degree of the ~ 87 kDa species (Fig. 4a and e). The addition of L-NAME and AG inhibited significantly the total levels of phospho-PKA substrates and particularly the ~ 87 kDa band (Fig. 4a, d and e). On the other hand, the ~ 62 kDa was not affected by any treatment (Fig. 4a and e). The simultaneous supplementation with L-Arginine and FF did not modify global phospho-PKA substrates, nor the ~ 62 kDa species, nevertheless the ~ 87 kDa band was affected by the NOS inhibitors, which diminished its amount of signal (Fig. 5a, d and e).

Total phosphorylation degree of tyrosine residues was found to be significantly lower than CONTROL and GSNO, when using NOS inhibitors (Fig 2b and f). Specifically, this was observed in the band of ~ 110 kDa, whereas in the ~ 75 kDa species this effect was evident only in the L-NAME group (Fig. 2b and g). In the presence of L-Arginine, L-NAME's inhibitory effect was maintained for both total and band-specific Tyr-P levels (Fig. 3b, f and g), especially vs. CONTROL. AG decreased Tyr-P when examining total phosphorylation levels and the ~ 110 kDa band, compared to CONTROL, although its effect was not as noticeable as with L-NAME (Fig. 3b, f and g). When

examining the FF supplementation, NOS inhibitors decreased significantly both global and the ~ 110 kDa Tyr-P levels, but no effect was evidenced in the ~ 75 kDa species (Fig. 4b, f and g). Furthermore, during the co-incubation with L-Arginine and FF, L-NAME led to lower overall and band-specific Tyr-P levels, particularly when compared to CONTROL (Fig. 5b, f and g). Under the same experimental conditions, AG also inhibited the total Tyr-P levels (Fig. 5b and f).

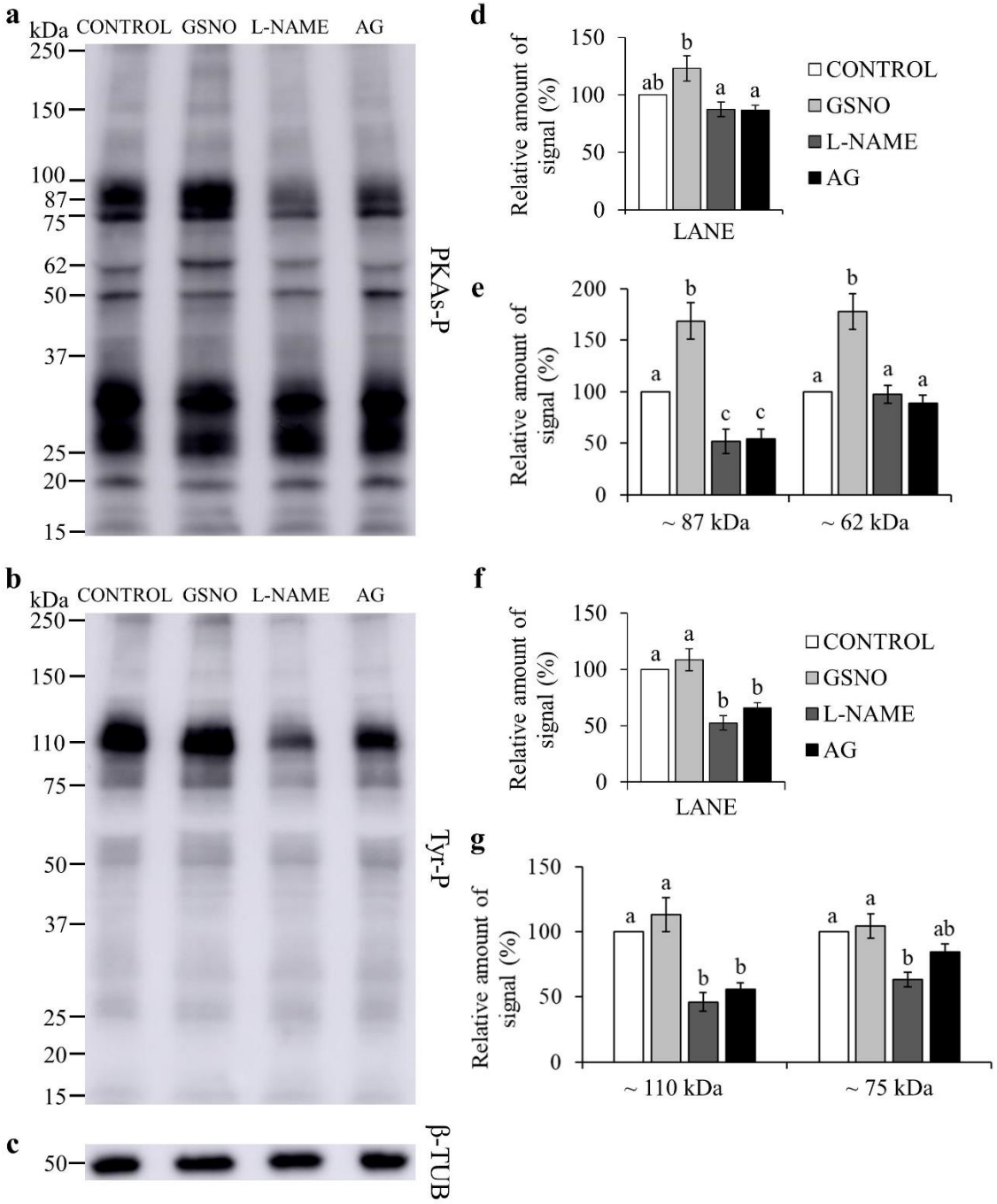


Figure 2. Effect of GSNO, L-NAME and AG on PKA substrates (PKAs-P) and tyrosine phosphorylation (Tyr-P). Sperm were incubated for 4 hours under capacitating conditions in the absence of any treatments (CONTROL) or in the presence of GSNO, a NO donor, and L-NAME and AG (both NOS inhibitors). (**a** and

b) Sperm protein extracts were analyzed for phosphorylation by Western blot using anti-PKAs-P or anti-Tyr-P as first antibodies, respectively. (**c**) β-tubulin (β-TUB) was used as a protein loading control. For signal quantification, each lane was normalized to its β-TUB optical density value. (**d-g**) Relative amount of signal quantified in each membrane using ImageQuant TL v8.1 software for PKAs-P and Tyr-P, respectively. Different letters (a, b, c) indicate statistically significant differences ($P<0.05$) between groups.

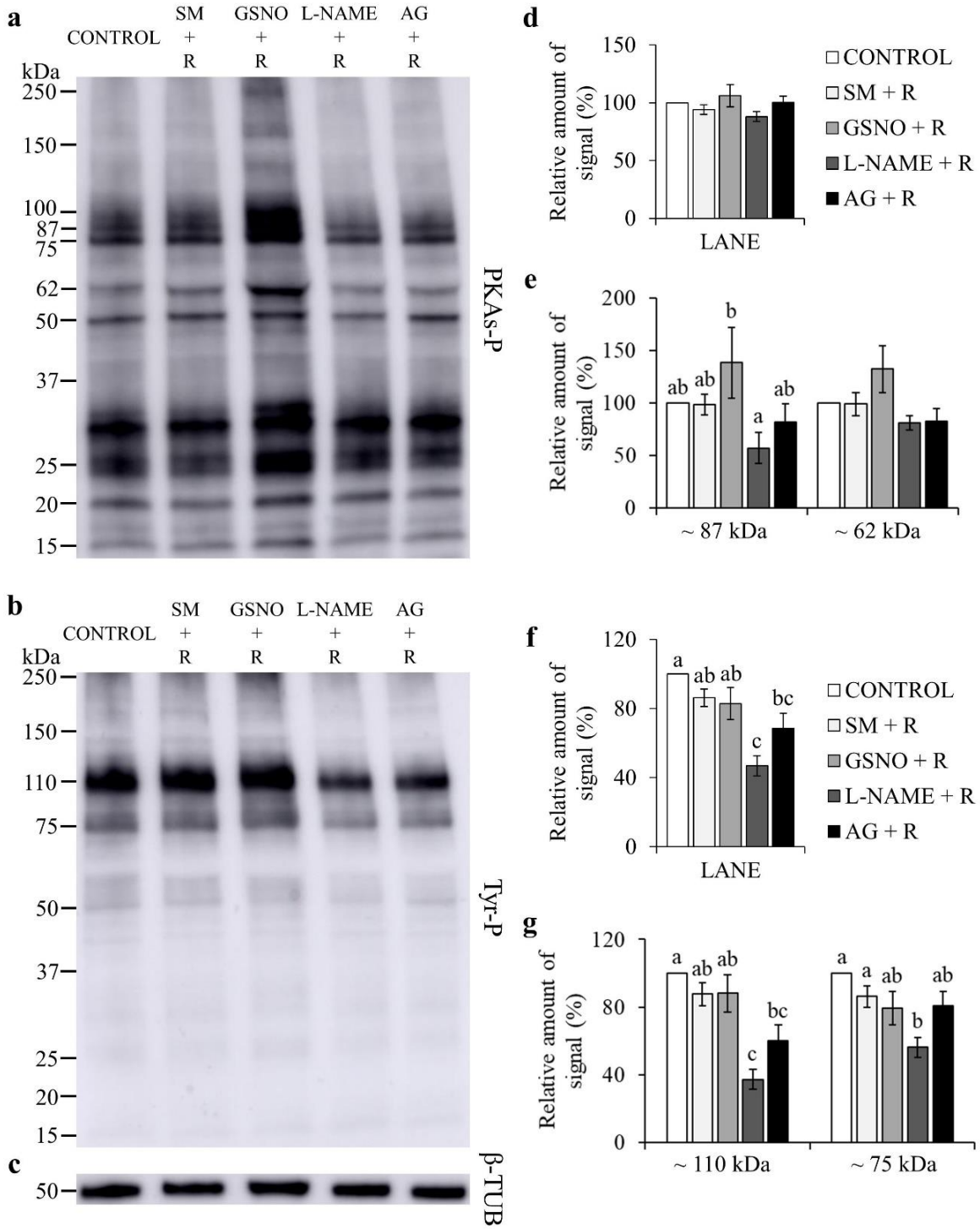


Figure 3. Effect of L-Arginine (R), GSNO, L-NAME and AG on PKA substrates (PKAs-P) and tyrosine phosphorylation (Tyr-P). Sperm were incubated for 4 hours under capacitating conditions in the absence of

any treatments (CONTROL) or in the presence of L-Arginine, the substrate for NO synthesis, GSNO, a NO donor, L-NAME and AG (both NOS inhibitors). (**a** and **b**) Sperm protein extracts were analyzed for phosphorylation by Western blot using anti-PKAs-P or anti-Tyr-P as first antibodies, respectively. (**c**) β-tubulin (β-TUB) was used as a protein loading control. For signal quantification, each lane was normalized to its β-TUB optical density value. (**d-g**) Relative amount of signal quantified in each membrane using ImageQuant TL v8.1 software for PKAs-P and Tyr-P, respectively. SM: Sperm Medium. Different letters (a, b, c) indicate statistically significant differences (P<0.05) between groups.

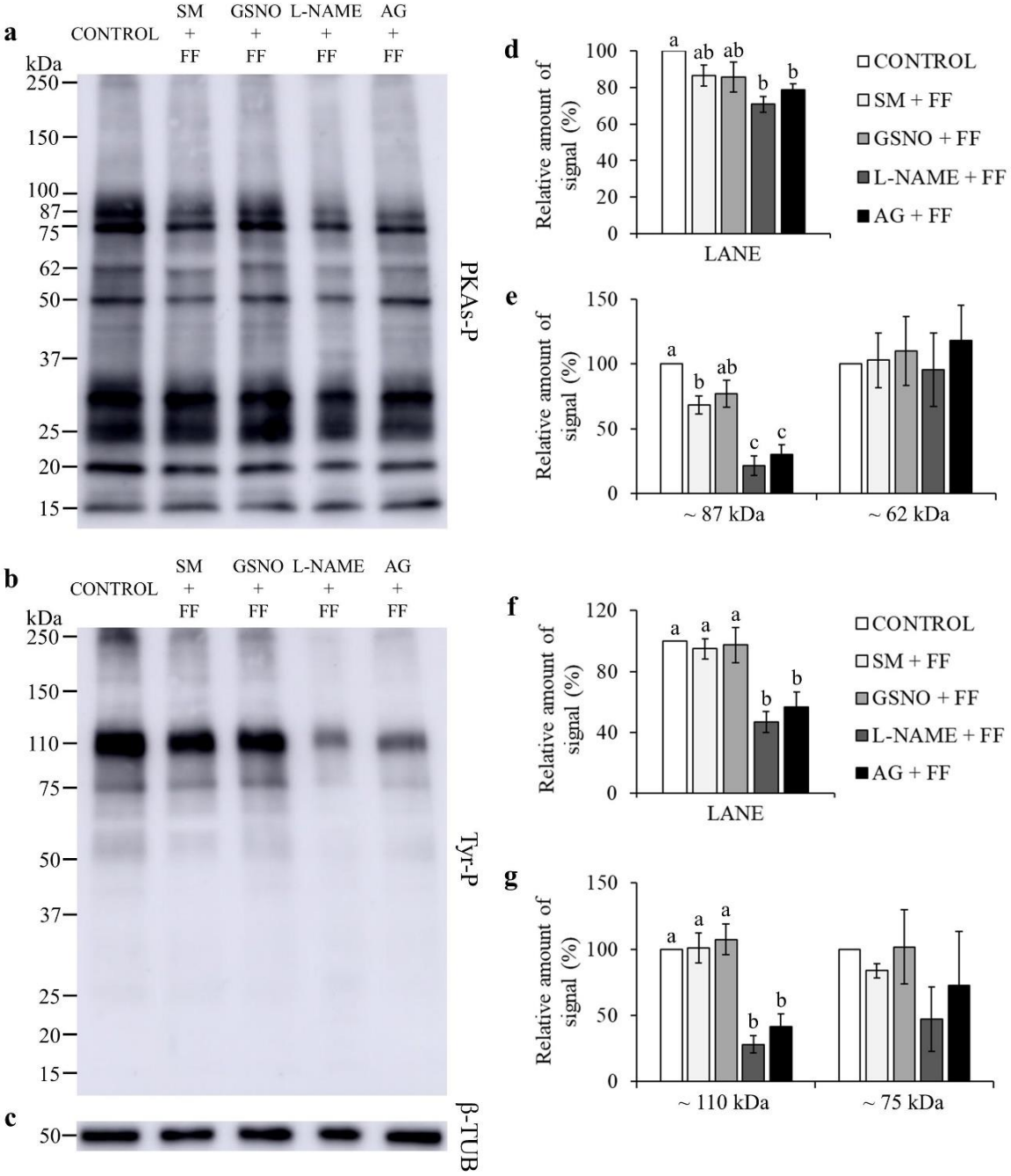


Figure 4. Effect of follicular fluid (FF), GSNO, L-NAME, and AG on PKA substrates (PKAs-P) and tyrosine phosphorylation (Tyr-P). Sperm were incubated for 4 hours under capacitating conditions in the

absence of any treatments (CONTROL) or in the presence of follicular fluid, GSNO, a NO donor, L-NAME and AG (both NOS inhibitors). (**a** and **b**) Sperm protein extracts were analyzed for phosphorylation by Western blot using anti-PKAs-P or anti-Tyr-P as first antibodies, respectively. (**c**) β-tubulin (β-TUB) was used as a protein loading control. For signal quantification, each lane was normalized to its β-TUB optical density value. (**d-g**) Relative amount of signal quantified in each membrane using ImageQuant TL v8.1 software for PKAs-P and Tyr-P, respectively. SM: Sperm Medium. Different letters (a, b, c) indicate statistically significant differences ($P<0.05$) between groups.

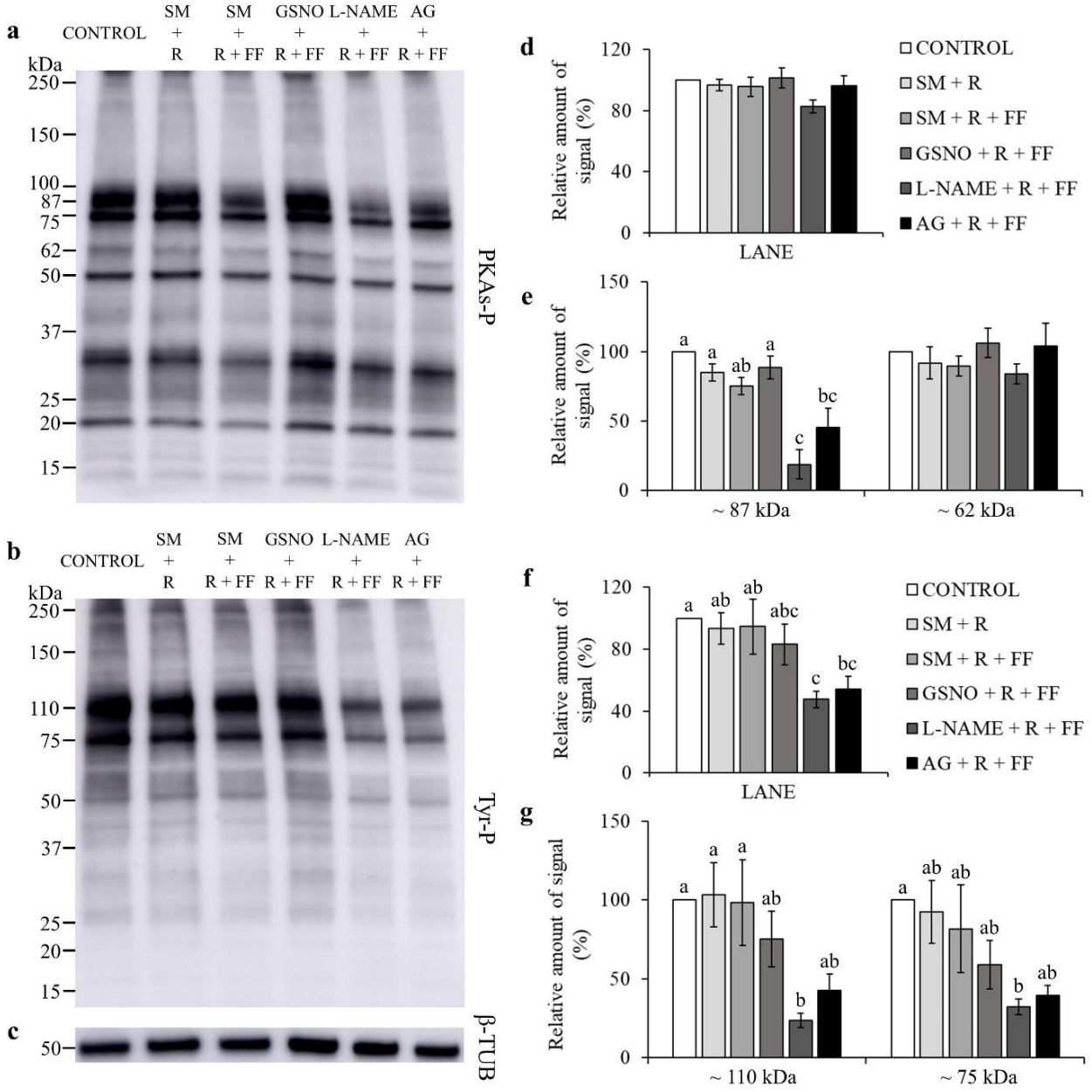


Figure 5. Effect of L-Arginine (R), follicular fluid (FF), GSNO, L-NAME, and AG on PKA substrates (PKAs-P) and tyrosine phosphorylation (Tyr-P). Sperm were incubated for 4 hours under capacitating conditions in the absence of any treatments (CONTROL) or in the presence of L-Arginine, the substrate for NO synthesis, follicular fluid, GSNO, a NO donor, L-NAME and AG (both NOS inhibitors). (**a** and **b**) Sperm

protein extracts were analyzed for phosphorylation by Western blot using anti-PKAs-P or anti-Tyr-P as first antibodies, respectively. (**c**) β-tubulin (β-TUB) was used as a protein loading control. For signal quantification, each lane was normalized to its β-TUB optical density value. (**d-g**) Relative amount of signal quantified in each membrane using ImageQuant TL v8.1 software for PKAs-P and Tyr-P, respectively. SM: Sperm Medium. Different letters (a, b, c) indicate statistically significant differences ($P<0.05$) between groups.

Mass spectrometry analysis

Four protein bands (~ 110, ~ 87, ~ 75 and ~ 62 kDa) were selected once the immunoblots were analyzed. After HPLC-ESI-Q-TOF-MS/MS analysis, a total of 29 proteins were identified (Table 1). The actual molecular weights ranged from 100.2 to 117.8 kDa for the ~ 110 kDa band, between 83.3 and 94.7 kDa for the ~ 87 kDa species, between 70 and 78.8 kDa for the ~ 75 kDa band and from 57.9 to 62.1 kDa in the case of the ~ 62 kDa species.

These proteins belong to different functional groups (Fig. 6), as follows: *spermatogenesis* (outer dense fiber of sperm tails 2, isoform CRA_c; heat shock 70kDa protein 2, isoform CRA_a; mitochondria-eating protein), *binding to the ZP* (T-complex protein 1 subunits gamma, alpha, theta and eta), *energy and metabolism* (hexokinase 1, isoform CRA_c; neutral alpha-glucosidase AB; transitional endoplasmic reticulum ATPase; ATP-dependent 6-phosphofructokinase, platelet type; alpha-1,4 glucan phosphorylase; glutamine--fructose-6-phosphate aminotransferase [isomerizing] 1; pyruvate kinase; pyruvate kinase PKM), *stress response* (epididymis luminal secretory protein 52; heat shock protein 90kDa alpha (Cytosolic), class B member 1, isoform CRA_a; transitional endoplasmic reticulum ATPase; endoplasmic reticulum chaperone BiP; heat shock 70kDa protein 2, isoform CRA_a), *motility and structural organization* (actinin, alpha 1, isoform CRA_a; endoplasmin; A kinase (PRKA) anchor protein 4, isoform CRA_c; A-kinase anchor protein 3; outer dense fiber of sperm tails 2, isoform CRA_c; T-complex protein 1 subunit alpha; WD repeat-containing protein 1), *signaling and protein turnover* (26S proteasome non-ATPase regulatory subunit 2; testicular secretory protein Li 63; heat shock protein 90kDa alpha (Cytosolic), class B member 1, isoform CRA_a; endoplasmin; A kinase (PRKA) anchor protein 4, isoform CRA_c; A-kinase anchor protein 3; transitional endoplasmic reticulum ATPase; endoplasmic reticulum chaperone BiP; heat shock 70kDa protein 2, isoform CRA_a; lactoferrin; T-complex protein 1 subunits gamma, theta and eta; epididymis secretory sperm binding protein; chaperonin containing TCP1, subunit 6A (Zeta 1), isoform CRA_a; mitochondria-eating protein).

Table 1. List of proteins identified via HPLC-ESI-Q-TOF-MS/MS in human spermatozoa.

Band (kD)	Accession No. (UniProtKB)	Protein name	Gene name	Molecular weight (kD)	No. of peptides	Sequence coverage (%)[1]	Biological functions
~ **110**	A0A024QZK7	Hexokinase 1, isoform CRA_c	HK1	102.7	15	15.9	Glycolytic process; glucose homeostasis; fructose metabolism.
	F5H6X6	Neutral alpha-glucosidase AB	GANAB	112.9	5	5.5	Carbohydrate metabolism.
	Q13200	26S proteasome non-ATPase regulatory subunit 2	PSMD2	100.2	3	3.5	Proteasome-mediated ubiquitin-dependent protein catabolism; MAPK cascade; post-translational protein modification.
	A0A024R694	Actinin, alpha 1, isoform CRA_a	ACTN1	103.1	3	3.3	Actin filament bundle assembly; actin crosslink formation.
	A0A024R1A3	Testicular secretory protein Li 63	UBE1	117.8	3	3.3	Ubiquitin activating enzyme activity.
~ **87**	K9JA46	Epididymis luminal secretory protein 52	EL52	84.7	39	49.5	Protein folding; response to cold or heat.
	A0A024RD80	Heat shock protein 90kDa alpha (Cytosolic), class B member 1, isoform CRA_a	HSP90AB1	83.3	33	42.1	Protein kinase regulator activity; regulation of proteasomal protein catabolic process; placenta development; regulation of cyclin-dependent protein kinase activity; regulation of peptidyl-serine phosphorylation.
	P14625	Endoplasmin	HSP90B1	92.5	10	13.2	Actin rod assembly; regulation of apoptotic process; post-translational protein modification; regulation of phosphoprotein phosphatase activity; sequestering of calcium ions.
	A0A384MQY7	A kinase (PRKA) anchor protein 4, isoform CRA_c	AKAP4	94.5	23	29.6	Protein binding and localization; sperm motility.
	O75969	A-kinase anchor protein 3	AKAP3	94.7	9	12.8	Protein binding and localization; sperm motility; capacitation; acrosome reaction; blastocyst hatching; regulation of protein serine/threonine kinase signaling pathway.
	P55072	Transitional endoplasmic reticulum ATPase	VCP	89.3	8	13.5	Cellular response to DNA damage stimulus and heat; flavin adenine dinucleotide catabolic process; NADH metabolic process; mitotic spindle disassembly; regulation of

Table 1 (continued)

Band (kD)	Accession No. (UniProtKB)	Protein name	Gene name	Molecular weight (kD)	No. of peptides	Sequence coverage (%)[1]	Biological functions
							mitochondrial membrane potential and oxidative phosphorylation; protein folding.
	Q01813	ATP-dependent 6-phosphofructokinase, platelet type	PFKP	85.6	5	7.6	Glycolysis; fructose 1,6-bisphosphate and fructose 6-phosphate metabolic process.
	B4DSD8	Alpha-1,4 glucan phosphorylase	*N/A*	85.8	3	5	Carbohydrate metabolism.
~ 75	A0A024R8A4	Outer dense fiber of sperm tails 2, isoform CRA_c	ODF2	73.3	22	32.4	Cilium organization; spermatid development.
	P11021	Endoplasmic reticulum chaperone BiP	HSPA5	72.3	21	36.8	Cellular response to cAMP, calcium ions, heat, drugs, radiation and unfolded proteins; protein ubiquitination; regulation of apoptotic process.
	A0A024R6B5	Heat shock 70kDa protein 2, isoform CRA_a	HSPA2	70.0	14	22.8	Male meiosis I; regulation of protein phosphorylation; response to cold or heat; spermatid development.
	A0A161I202	Lactoferrin	LTF	78.3	9	15.6	Regulation of protein serine/threonine kinase activity; regulation of ATPase activity, membrane potential and apoptotic process.
	Q06210	Glutamine--fructose-6-phosphate aminotransferase [isomerizing] 1	GFPT1	78.8	5	7	Glutamine and fructose 6-phosphate metabolism; protein N-linked glycosylation.
~ 62	A0A024R5Z9	Pyruvate kinase	PKM2	58.1	23	44.4	ATP biosynthesis; glucose metabolism.
	P14618	Pyruvate kinase PKM	PKM	57.9	22	47	ATP biosynthesis; glucose metabolism.
	P49368	T-complex protein 1 subunit gamma	CCT3	57.9	12	28.5	Binding of sperm to zona pellucida; protein folding.
	P17987	T-complex protein 1 subunit alpha	TCP1	60.3	13	24.4	Binding of sperm to zona pellucida; protein folding; tubulin complex assembly.
	P50990	T-complex protein 1 subunit theta	CCT8	59.6	6	13.6	Binding of sperm to zona pellucida; protein folding.
	A0A024R3X4	Epididymis secretory sperm binding protein	HSPD1	61.1	6	10.2	De novo protein folding; protein refolding; mitochondrion organization; regulation of apoptotic process.
	B3KN28	Phosphoacetylglucosamine mutase	*N/A*	59.9	4	6	Carbohydrate metabolism; UDP-N-acetylglucosamine biosynthetic process.

Table 1 (continued)

Band (kD)	Accession No. (UniProtKB)	Protein name	Gene name	Molecular weight (kD)	No. of peptides	Sequence coverage (%)[1]	Biological functions
	Q99832	T-complex protein 1 subunit eta	CCT7	59.3	3	5.1	Binding of sperm to zona pellucida; protein folding.
	O75083	WD repeat-containing protein 1	WDR1	62.1	3	8	Actin filament depolymerization and fragmentation.
	A0A024RDL1	Chaperonin containing TCP1, subunit 6A (Zeta 1), isoform CRA_a	CCT6A	58.0	3	8	Protein folding.
	Q8TC71	Mitochondria-eating protein	SPATA18	61.1	2	4.4	Mitochondrial protein catabolism; spermatogenesis.

[1]Percentage of the protein sequence covered by identified peptides.

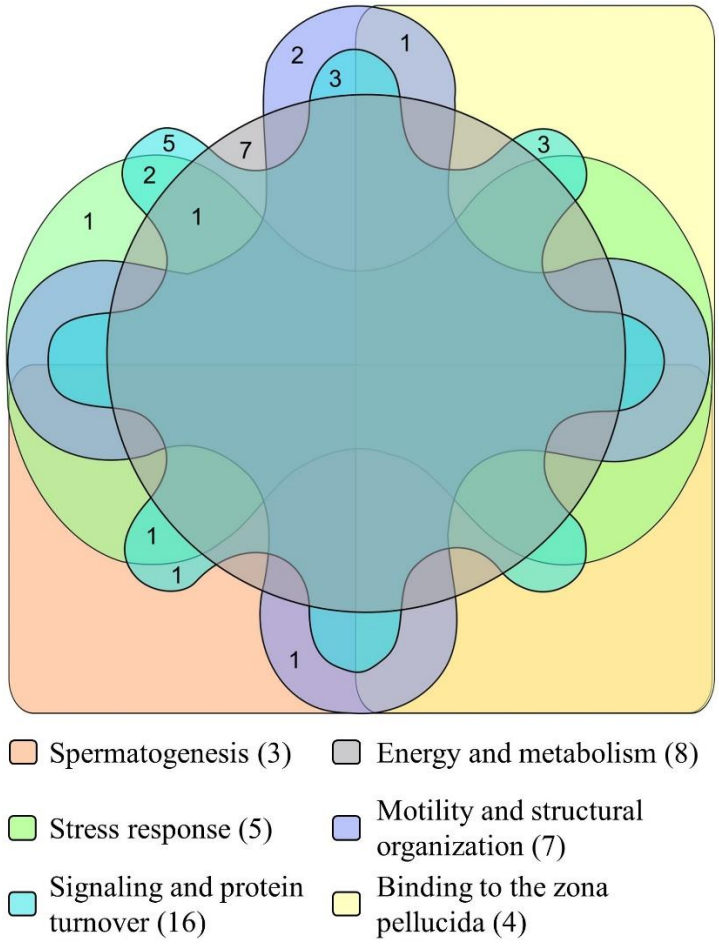


Figure 6. Venn diagram illustrating different functional groups for the proteins identified via HPLC-ESI-Q-TOF-MS/MS. The figure was created using the web-based tool InteractiVenn [79].

4. DISCUSSION

For the gamete interaction to be successful during fertilization, both sperm and oocytes need to undergo structural and functional changes, which are, in part, mediated by NO [35]. Particularly, for spermatozoa, these physiological modifications are globally known as capacitation [36]. Although many years have passed since sperm capacitation was first reported, the molecular basis of this process is still not completely understood [11]. NO emerged as one the messengers involved in regulating protein phosphorylation levels [14,37,38]. In the present paper, we provide more evidence on how NO modulates this aspect.

Our results showed an increase in the phosphorylation degree of two PKA substrates, namely ~ 87 kDa and ~ 62 kDa, when using GSNO. Similarly, Rahman *et al*. [38] reported that sodium nitroprusside, a NO-releasing agent, increased the levels of different PKA substrate species in mouse spermatozoa. On the other hand, we did not observe any effect of GSNO on the total levels of Tyr-P nor on the bands of ~ 110 kDa and ~ 75 kDa. This contrasts previous studies, where the authors

described an increase in Tyr-P when using NO donors [14] in specific human [37] and mouse sperm proteins [38]. We hypothesize that other molecules might have been responsible for Tyr-P in our experiment, therefore, concealing any effect of GSNO. In fact, it has been shown that the superoxide anion and hydrogen peroxide, which are endogenously generated by spermatozoa, can also induce Tyr-P and capacitation [39–41].

When we inhibited NO synthesis, especially with L-NAME, the phosphorylation degree of PKA substrates and tyrosine residues decreased significantly. Our observations agree with other reports, in which NOS inhibitors suppressed Tyr-P in human [14,37] and buffalo [42] sperm proteins. Interestingly, the phosphorylation level of the ~ 87 kDa band was lowered by the NOS inhibitors, even when compared to the CONTROL group. This result clearly suggests that NO mediates the phosphorylation of this species and this might be an important step during human sperm capacitation. Furthermore, the NO-triggered phosphorylation of this species by PKA, on serine and threonine residues, might be required prior to its phosphorylation on tyrosine residues [15]. This hypothesis should be tested in future studies.

Previous studies found that L-Arginine, a NO precursor, is a capacitating agent and L-NAME reduces the L-Arginine-induced capacitation in buffalo and bovine spermatozoa [42,43]. Moreover, Thundathil *et al*. [37] observed that L-Arginine caused an increase in a threonine-glutamine-tyrosine motif in two different human sperm proteins and this effect was prevented by L-NAME. Similar results were described by Roy and Atreja [42] when analyzing Tyr-P. In contrast to these works, we did not find any differences in the total levels of phospho-PKA substrates and Tyr-P, when adding L-Arginine to the capacitation medium. The reason for this could be that present levels of NO may have been sufficient to induce sperm protein phosphorylation [44]. However, the simultaneous presence of L-Arginine and L-NAME did not alter L-NAME's inhibitory effect on total Tyr-P and phosphotyrosine levels of the ~ 110 kDa and ~ 75 kDa bands. We also observed that when L-Arginine was used, the effect of AG on Tyr-P was not as marked as with L-NAME, which could be attributed to AG's ability to selectively inhibit one of three NOS isoforms, responsible for NO production [45]. Therefore, we might assume that L-Arginine might have partially reverted AG's effect, compared to L-NAME.

Since *in vitro* fertilization techniques make easy the recovery of human follicular fluid, several studies aimed to determine its effects on human sperm function (summarized by [46]). Gye MC [47] reported that FF increased Tyr-P levels of the ~ 56 kDa band in mouse epididymal sperm. On the contrary, we did not observe a significant effect of FF on Tyr-P, neither global nor band-specific. Our results might be explained by species differences and/or by the use of epidydimal vs ejaculated

spermatozoa [48]. Our observations agree, however, with a previous work in which no modification in the Tyr-P signal of human sperm was observed in the presence of FF, even though this fluid was used at a higher concentration [46]. Interestingly, the ~ 87 kDa species exhibited a lower degree of phosphorylation in the presence of FF. Munuce *et al*. [46] reported a reduction in the number of human spermatozoa bound to hemizonas after being exposed to FF, which was associated with a significant loss in mannose-binding sites in these cells. The latter have been proposed as ZP recognition molecules [49]. Certain glycoproteins in the FF seem to inhibit sperm binding to ZP [50,51], possibly by masking the mannose residues on sperm surface [46,52]. For these reasons, the phosphorylation to a lesser extent of the ~ 87 kDa band, observed in the presence of FF in our study, might be important for the correct distribution of mannose-binding sites in spermatozoa. The FF supplementation did not revert the NOS inhibitors' effect in our study, which lowered, also in this case, the levels of phospho-PKA substrates and Tyr-P. Furthermore, we observed that during the co-incubation with L-Arginine and FF, spermatozoa continued to display low Tyr-P levels in the presence of NOS inhibitors, particularly with L-NAME. However, although global phosphorylation levels of PKA substrates did not exhibit changes under these conditions, the ~ 87 kDa band presented a low phosphorylation degree, which clearly suggests its importance during sperm capacitation.

Our data confirm that NO is a very important mediator of phosphorylation events during capacitation, particularly, the presence/absence of NO seemed to affect certain WB bands more than others. Therefore, we proceeded to the identification of the proteins in said bands and divided them in 6 groups depending on their function/s.

Some of these proteins (i.e. outer dense fiber of sperm tails 2, isoform CRA_c; heat shock 70kDa protein 2, isoform CRA_a and the mitochondria-eating protein) are known to be involved in the spermatogenesis [53–55], even though they possess overlapping functions with other functional groups. Particularly, the expression of the heat shock 70 kDa protein 2 takes place during the early phases of meiosis I [56,57] and the ablation of the gene encoding this protein evidenced its importance during spermatogenesis [58]. Interestingly, mature spermatozoa retain this protein and it has been postulated that it may assist the assembly, repositioning and/or unmasking of ZP recognition complexes on the surface of spermatozoa [54].

Other proteins, such as the T-complex protein 1 subunits alpha, gamma, eta and theta are known components of the chaperonin-containing TCP-1 complex [59]. A previous study [60] showed how the latter is involved in reorganizing the cytoskeleton during the differentiation of haploid spermatids into spermatozoa. Moreover, mouse spermatozoa express the TCP-1 complex in the apical region of their head, from where it may modulate interactions with the ZP [59].

Another functional group of proteins in our study concerns energy production. To sustain their metabolism, spermatozoa require adenosine triphosphate (ATP), which can be obtained via glycolysis (mainly in the fibrous sheath of the flagellum) and oxidative phosphorylation (during mitochondrial respiration, in the midpiece) [61]. Spermatozoa synthesize ATP from different substrates, such as glucose, pyruvate and lactate, but the preferred metabolic pathway for ATP production during sperm capacitation depends on the species [62,63].

Some heat shock proteins, belonging to the 90 kDa and 70kDa families, were also identified in our study. These chaperones are known regulators of apoptosis, particularly, they can either induce or inhibit this process during spermatogenesis, to guarantee the elimination of abnormal sperm cells and the maintenance of an adequate number of germ cells [64]. Moreover, evidence suggests that heat shock proteins commonly undergo S-nitrosylation in human sperm and can be tyrosine phosphorylated during capacitation [12].

The predominant functions of the proteins identified in the motility and structural organization group, concern either the regulation of sperm movement or the actin filament assembly/depolymerization. For instance, the A-kinase anchoring protein 3 and 4 were described in the fibrous sheath of the sperm flagellum, where they can bind phosphodiesterase isoforms or the regulatory subunit of PKA (summarized by [12]), and consequently, regulate sperm motility [65]. Furthermore, it was demonstrated that they are subjected to S-nitrosylation, which is confirmed by the presence of an S-nitrosylation motif in their primary sequences [12]. The outer dense fiber 2 proteins and similar polypeptides surround the axoneme and form a key cytoskeletal structure in the sperm tail, called the outer dense fiber sheath [66–68]. Their role in ensuring a physiological sperm movement was evidenced in asthenozoospermic men, who showed an abnormal expression and distribution of these proteins [68,69]. Furthermore, the actin cytoskeleton undergoes a dynamic remodeling during sperm capacitation [70] and, in this sense, Finkelstein *et al*. [71] showed how actin polymerization increases in the head of capacitated human spermatozoa. This phenomenon is believed to prevent the spontaneous acrosome reaction by creating a physical barrier between the outer acrosomal membrane and the plasma membrane [72].

A considerable amount of the proteins identified in our WB bands are involved in signaling pathways, which is not surprising since their fine-tuning is essential for sperm to acquire their fertilizing ability. The cAMP/PKA, cGMP/PKG and the extracellular-regulated kinase pathways trigger highly compartmentalized phosphorylation events of proteins which govern sperm motility, viability, hyperactivation and acrosome reaction [11,73]. These are not, however, the only post-translational modifications that take place in spermatozoa. In fact, protein ubiquitination is also

relevant for sperm physiology [74]. In particular, apart from targeting proteins for degradation during spermiogenesis [75], the ubiquitination may be involved in eliminating defective spermatozoa in the epididymis [76,77]. Additionally, sperm mitochondria may be ubiquitinated and, therefore, tagged for destruction later in the zygote [78].

5. ACKNOWLEDGEMENTS

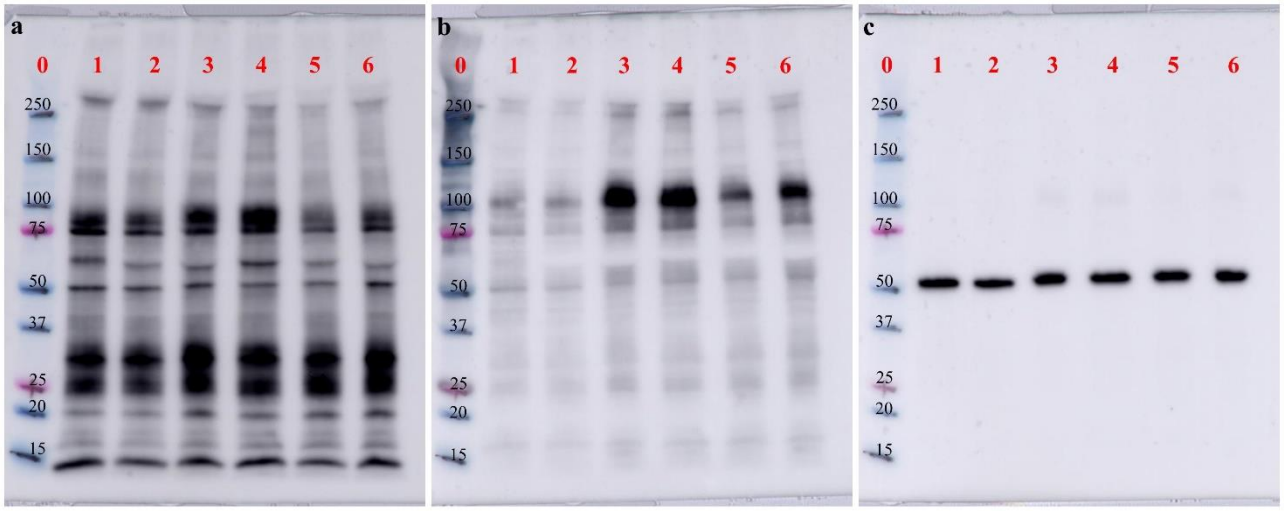
The authors involved in this work would like to thank all the staff from the Andrology and Embryology laboratories at IVI-RMA Global (Murcia, Spain) and Analuce Canha-Gouveia for their assistance in recruiting the semen and FF donors. Furthermore, the authors thank Prof. Javier Corral de la Calle for providing the HemoCue Plasma/Low Hb System and Dr. Alejandro Torrecillas for performing the HPLC-ESI-Q-TOF-MS/MS analysis.

6. SUPPORTING INFORMATION

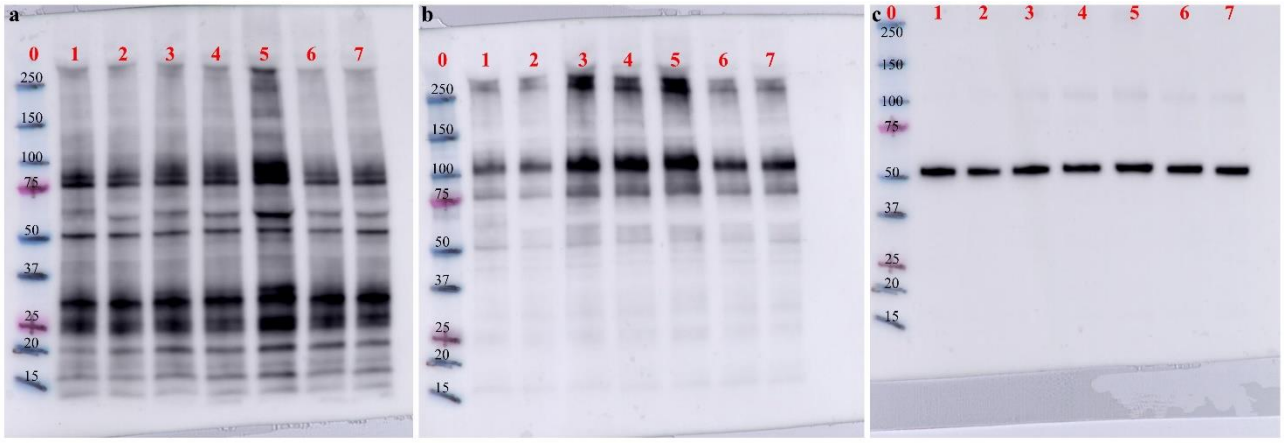
Supplementary table. Hemoglobin levels in follicular fluid samples.

FF sample	Hb before processing (g/dL)	Hb after processing (g/dL)
1	0.02 (0.00)	0.00 (0.00)
2	0.02 (0.00)	0.00 (0.00)
3	0.00 (0.00)	0.00 (0.00)
4	0.02 (0.00)	0.00 (0.00)
5	0.02 (0.00)	0.00 (0.00)
6	0.01 (0.00)	0.00 (0.00)
7	0.04 (0.00)	0.00 (0.00)
8	0.10 (0.00)	0.00 (0.00)
9	0.00 (0.00)	0.00 (0.00)
10	0.00 (0.00)	0.00 (0.00)
11	0.00 (0.00)	0.00 (0.00)
12	0.03 (0.00)	0.00 (0.00)
13	0.02 (0.00)	0.00 (0.00)
14	0.02 (0.00)	0.00 (0.00)
15	0.06 (0.00)	0.00 (0.00)
16	0.03 (0.00)	0.00 (0.00)
17	0.02 (0.00)	0.00 (0.00)
18	0.02 (0.00)	0.00 (0.00)
19	0.08 (0.00)	0.00 (0.00)
20	0.00 (0.00)	0.00 (0.00)
21	0.02 (0.00)	0.00 (0.00)
22	0.00 (0.00)	0.00 (0.00)
23	0.05 (0.00)	0.00 (0.00)
24	0.00 (0.00)	0.00 (0.00)
25	0.04 (0.00)	0.00 (0.00)
26	0.00 (0.00)	0.00 (0.00)

FF, follicular fluid; Hb, hemoglobin; measurements were performed in duplicate and results are represented as mean (SD).

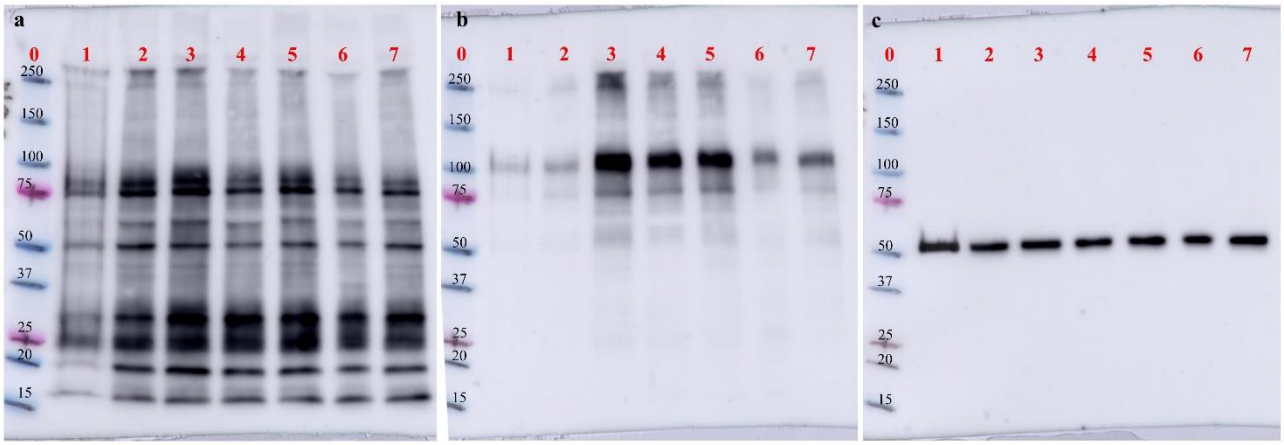


Supplementary file for Figure 2. Representative membranes for phospho-PKA substrates (a), tyrosine phosphorylation (b) and β-tubulin (c). Red numbers indicate the content of each lane. (**0**) Precision Plus Protein™ Dual Color Standards (Bio-Rad, Madrid, Spain, #1610374). Molecular weights are expressed in kilodaltons. (**1-6**) Immunoblotted protein extracts from spermatozoa incubated under different experimental conditions, as follows: (**1**) Dulbecco's Phosphate-Buffered Saline without calcium chloride and magnesium chloride, time 0 h. (**2**) Sperm medium, time 0 h. (**3**) CONTROL: sperm medium, time 4 h. (**4**) GSNO: sperm medium supplemented with 100 µM S-Nitrosoglutathione, time 4 h. (**5**) L-NAME: sperm medium supplemented with 10 mM N^G-Nitro-L-Arginine Methyl Ester Hydrochloride, time 4 h. (**6**) AG: sperm medium supplemented with 10 mM Aminoguanidine Hemisulfate salt, time 4 h.

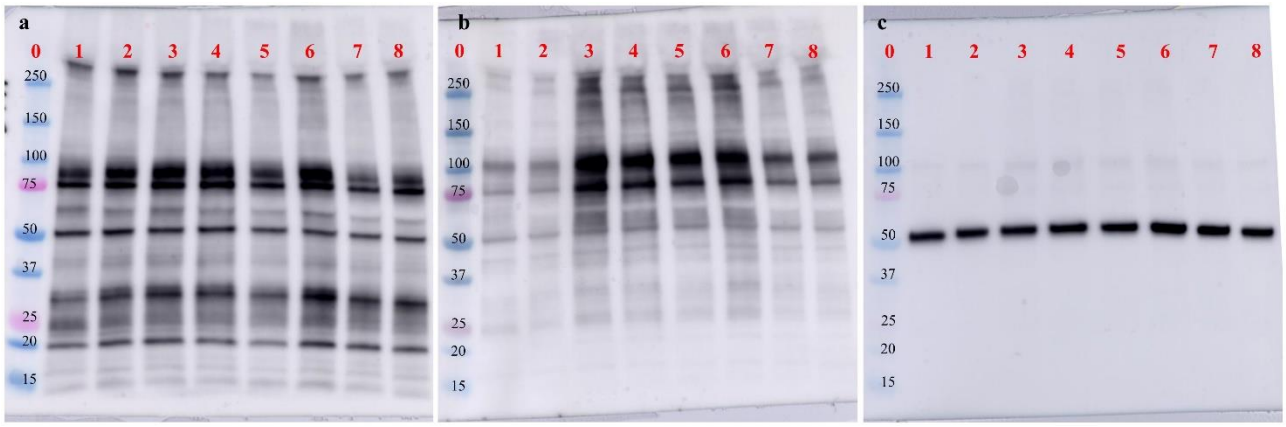


Supplementary file for Figure 3. Representative membranes for phospho-PKA substrates (a), tyrosine phosphorylation (b) and β-tubulin (c). Red numbers indicate the content of each lane. (**0**) Precision Plus Protein™ Dual Color Standards (Bio-Rad, Madrid, Spain, #1610374). Molecular weights are expressed in kilodaltons. (**1-7**) Immunoblotted protein extracts from spermatozoa incubated under different experimental conditions, as follows: (**1**) Dulbecco's Phosphate-Buffered Saline without calcium chloride and magnesium chloride, time 0 h. (**2**) Sperm medium, time 0 h. (**3**) CONTROL: sperm medium, time 4 h. (**4**) SM + R: sperm medium supplemented with 10 mM L-Arginine, time 4 h. (**5**) GSNO + R: sperm medium supplemented with

100 µM S-Nitrosoglutathione and 10 mM L-Arginine, time 4 h. (**6**) L-NAME + R: sperm medium supplemented with 10 mM N^G-Nitro-L-Arginine Methyl Ester Hydrochloride and 10 mM L-Arginine, time 4 h. (**7**) AG + R: sperm medium supplemented with 10 mM Aminoguanidine Hemisulfate salt and 10 mM L-Arginine, time 4 h.



Supplementary file for Figure 4. Representative membranes for phospho-PKA substrates (a), tyrosine phosphorylation (b) and β-tubulin (c). Red numbers indicate the content of each lane. (**0**) Precision Plus Protein™ Dual Color Standards (Bio-Rad, Madrid, Spain, #1610374). Molecular weights are expressed in kilodaltons. (**1-7**) Immunoblotted protein extracts from spermatozoa incubated under different experimental conditions, as follows: (**1**) Dulbecco's Phosphate-Buffered Saline without calcium chloride and magnesium chloride, time 0 h. (**2**) Sperm medium, time 0 h. (**3**) CONTROL: sperm medium, time 4 h. (**4**) SM + FF: sperm medium supplemented with 20% *v/v* follicular fluid, time 4 h. (**5**) GSNO + FF: sperm medium supplemented with 100 µM S-Nitrosoglutathione and 20% *v/v* follicular fluid, time 4 h. (**6**) L-NAME + FF: sperm medium supplemented with 10 mM N^G-Nitro-L-Arginine Methyl Ester Hydrochloride and 20% *v/v* follicular fluid, time 4 h. (**7**) AG + FF: sperm medium supplemented with 10 mM Aminoguanidine Hemisulfate salt and 20% *v/v* follicular fluid, time 4 h.



Supplementary file for Figure 5. Representative membranes for phospho-PKA substrates (a), tyrosine phosphorylation (b) and β-tubulin (c). Red numbers indicate the content of each lane. (**0**) Precision Plus

Protein™ Dual Color Standards (Bio-Rad, Madrid, Spain, #1610374). Molecular weights are expressed in kilodaltons. (**1-8**) Immunoblotted protein extracts from spermatozoa incubated under different experimental conditions, as follows: (**1**) Dulbecco's Phosphate-Buffered Saline without calcium chloride and magnesium chloride, time 0 h. (**2**) Sperm medium, time 0 h. (**3**) CONTROL: sperm medium, time 4 h. (**4**) SM + R: sperm medium supplemented with 10 mM L-Arginine, time 4 h. (**5**) SM + R + FF: sperm medium supplemented with 10 mM L-Arginine and 20% *v/v* follicular fluid, time 4 h. (**6**) GSNO + R + FF: sperm medium supplemented with 100 µM S-Nitrosoglutathione, 10 mM L-Arginine and 20% *v/v* follicular fluid, time 4 h. (**7**) L-NAME + R + FF: sperm medium supplemented with 10 mM N^G-Nitro-L-Arginine Methyl Ester Hydrochloride, 10 mM L-Arginine and 20% *v/v* follicular fluid, time 4 h. (**8**) AG + R + FF: sperm medium supplemented with 10 mM Aminoguanidine Hemisulfate salt, 10 mM L-Arginine and 20% *v/v* follicular fluid, time 4 h.

7. REFERENCES

[1] Pacher P, Beckman JS, Liaudet L. Nitric Oxide and Peroxynitrite in Health and Disease. Physiol Rev 2007;87:315–424. doi:10.1152/physrev.00029.2006.

[2] Rosselli M, Keller PJ, Dubey RK. Role of nitric oxide in the biology, physiology and pathophysiology of reproduction. Hum Reprod Update 1998;4:3–24.

[3] Staicu F-D, Matas Parra C. Nitric Oxide: Key Features in Spermatozoa. In: Nitric Oxide Synthase - Simple Enzyme-Complex Roles, Seyed Soheil Saeedi Saravi, InTechOpen; 2017, p. 137–54. doi:10.5772/67551.

[4] O'Bryan MK, Zini A, Cheng CY, Schlegel PN. Human sperm endothelial nitric oxide synthase expression: correlation with sperm motility. Fertil Steril 1998;70:1143–7. doi:10.1016/S0015-0282(98)00382-3.

[5] Herrero MB, Pérez Martínez S, Viggiano JM, Polak JM, de Gimeno MF. Localization by indirect immunofluorescence of nitric oxide synthase in mouse and human spermatozoa. Reprod Fertil Dev 1996;8:931–4.

[6] Wang J, He Q, Yan X, Cai Y, Chen J. Effect of exogenous nitric oxide on sperm motility in vitro. Biol Res 2014;47:44. doi:10.1186/0717-6287-47-44.

[7] Zini A, De Lamirande E, Gagnon C. Low levels of nitric oxide promote human sperm capacitation in vitro. J Androl 1995;16:424–31.

[8] Herrero MB, Gagnon C. Nitric oxide: a novel mediator of sperm function. J Androl 2001;22:349–56.

[9] Revelli A, Soldati G, Costamagna C, Pellerey O, Aldieri E, Massobrio M, et al. Follicular fluid proteins stimulate nitric oxide (NO) synthesis in human sperm: a possible role for NO in acrosomal reaction. J Cell Physiol 1999;178:85–92. doi:10.1002/(SICI)1097-4652(199901)178:1<85::AID-JCP11>3.0.CO;2-Y.

[10] Donnelly ET, Lewis SE, Thompson W, Chakravarthy U. Sperm nitric oxide and motility: the effects of nitric oxide synthase stimulation and inhibition. Mol Hum Reprod 1997;3:755–62.

[11] Jin S-K, Yang W-X. Factors and pathways involved in capacitation: how are they regulated? Oncotarget 2017;8:3600–27. doi:10.18632/oncotarget.12274.

[12] Lefièvre L, Chen Y, Conner SJ, Scott JL, Publicover SJ, Ford WCL, et al. Human spermatozoa contain multiple targets for protein S-nitrosylation: An alternative mechanism of the modulation of sperm function by nitric oxide? Proteomics 2007;7:3066–84. doi:10.1002/pmic.200700254.

[13] Belén Herrero M, Chatterjee S, Lefièvre L, De Lamirande E, Gagnon C. Nitric oxide interacts with the cAMP pathway to modulate capacitation of human spermatozoa. Free Radic Biol Med 2000;29:522–36. doi:10.1016/S0891-5849(00)00339-7.

[14] Herrero MB, de Lamirande E, Gagnon C. Nitric oxide regulates human sperm capacitation and protein-tyrosine phosphorylation in vitro. Biol Reprod 1999;61:575–81. doi:10.1095/biolreprod61.3.575.

[15] Visconti PE, Westbrook VA, Chertihin O, Demarco I, Sleight S, Diekman AB. Novel signaling pathways involved in sperm acquisition of fertilizing capacity. J Reprod Immunol 2002;53:133–50.

[16] McVey M, Hill J, Howlett A, Klein C. Adenylyl cyclase, a coincidence detector for nitric oxide. J Biol Chem 1999;274:18887–92.

[17] Murad F. The nitric oxide-cyclic GMP signal transduction system for intracellular and intercellular communication. Recent Prog Horm Res 1994;49:239–48.

[18] Lohmann SM, Vaandrager AB, Smolenski A, Walter U, De Jonge HR. Distinct and specific functions of cGMP-dependent protein kinases. Trends Biochem Sci 1997;22:307–12.

[19] Pfeifer A, Ruth P, Dostmann W, Sausbier M, Klatt P, Hofmann F. Structure and function of cGMP-dependent protein kinases. Rev Physiol Biochem Pharmacol 1999;135:105–49.

[20] Revelli A, Costamagna C, Moffa F, Aldieri E, Ochetti S, Bosia A, et al. Signaling pathway of nitric oxide-induced acrosome reaction in human spermatozoa. Biol Reprod 2001;64:1708–12.

[21] Miraglia E, De Angelis F, Gazzano E, Hassanpour H, Bertagna A, Aldieri E, et al. Nitric oxide stimulates human sperm motility via activation of the cyclic GMP/protein kinase G signaling pathway. Reproduction 2011;141:47–54. doi:10.1530/REP-10-0151.

[22] Bender AT, Beavo JA. Cyclic Nucleotide Phosphodiesterases: Molecular Regulation to Clinical Use. Pharmacol Rev 2006;58:488–520. doi:10.1124/pr.58.3.5.

[23] Conti M, Beavo J. Biochemistry and physiology of cyclic nucleotide phosphodiesterases: essential components in cyclic nucleotide signaling. Annu Rev Biochem 2007;76:481–511. doi:10.1146/annurev.biochem.76.060305.150444.

[24] Beavo JA. Cyclic nucleotide phosphodiesterases: functional implications of multiple isoforms. Physiol Rev 1995;75:725–48. doi:10.1152/physrev.1995.75.4.725.

[25] Barroso G, Barrionuevo M, Rao P, Graham L, Danforth D, Huey S, et al. Vascular endothelial growth factor, nitric oxide, and leptin follicular fluid levels correlate negatively with embryo quality in IVF patients. Fertil Steril 1999;72:1024–6.

[26] Melo M, Busso CE, Bellver J, Alama P, Garrido N, Meseguer M, et al. GnRH agonist versus recombinant HCG in an oocyte donation programme: a randomized, prospective, controlled, assessor-blind study. Reprod Biomed Online 2009;19:486–92.

[27] Azarov I, Huang KT, Basu S, Gladwin MT, Hogg N, Kim-Shapiro DB. Nitric Oxide Scavenging by Red Blood Cells as a Function of Hematocrit and Oxygenation. J Biol Chem 2005;280:39024–32. doi:10.1074/jbc.M509045200.

[28] Soriano-Úbeda C, García-Vázquez FA, Romero-Aguirregomezcorta J, Matás C. Improving porcine in vitro fertilization output by simulating the oviductal environment. Sci Rep 2017;7:43616. doi:10.1038/srep43616.

[29] Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970;227:680–5.

[30] Romero-Aguirregomezcorta J, Santa ÁP, García-Vázquez FA, Coy P, Matás C. Nitric oxide synthase (NOS) inhibition during porcine in vitro maturation modifies oocyte protein S-nitrosylation and in vitro fertilization. PLoS One 2014;9:e115044. doi:10.1371/journal.pone.0115044.

[31] Leal ACMS, Caldas-Bussiere MC, Carvalho CSP de, Viana KS, Quirino CR. Role of nitric oxide on quality of freshly ejaculated bull spermatozoa during heparin-induced in vitro capacitation. Anim Reprod Sci 2009;116:38–49. doi:10.1016/j.anireprosci.2008.12.020.

[32] Calvo L, Vantman D, Banks SM, Tezon J, Koukoulis GN, Dennison L, et al. Follicular fluid-induced acrosome reaction distinguishes a subgroup of men with unexplained infertility not identified by semen analysis. Fertil Steril 1989;52:1048–54. doi:10.1016/S0015-0282(16)53173-2.

[33] Getpook C, Wirotkarun S. Sperm motility stimulation and preservation with various concentrations of follicular fluid. J Assist Reprod Genet 2007;24:425–8. doi:10.1007/s10815-007-9145-6.

[34] O'Flaherty C, de Lamirande E, Gagnon C. Positive role of reactive oxygen species in mammalian sperm capacitation: triggering and modulation of phosphorylation events. Free Radic Biol Med 2006;41:528–40. doi:10.1016/j.freeradbiomed.2006.04.027.

[35] Tosti E, Ménézo Y. Gamete activation: basic knowledge and clinical applications. Hum Reprod Update 2016;22:420–39. doi:10.1093/humupd/dmw014.

[36] Stival C, Puga Molina L del C, Paudel B, Buffone MG, Visconti PE, Krapf D. Sperm Capacitation and Acrosome Reaction in Mammalian Sperm. Adv. Anat. Embryol. Cell Biol., vol. 220, 2016, p. 93–106. doi:10.1007/978-3-319-30567-7_5.

[37] Thundathil J, de Lamirande E, Gagnon C. Nitric oxide regulates the phosphorylation of the threonine-glutamine-tyrosine motif in proteins of human spermatozoa during capacitation. Biol Reprod 2003;68:1291–8. doi:10.1095/biolreprod.102.008276.

[38] Rahman MS, Kwon W-S, Lee J-S, Kim J, Yoon S-J, Park Y-J, et al. Sodium nitroprusside suppresses male fertility in vitro. Andrology 2014;2:899–909. doi:10.1111/j.2047-2927.2014.00273.x.

[39] Aitken RJ, Paterson M, Fisher H, Buckingham DW, van Duin M. Redox regulation of tyrosine phosphorylation in human spermatozoa and its role in the control of human sperm function. J Cell Sci 1995;108 (Pt 5:2017–25.

[40] Leclerc P, de Lamirande E, Gagnon C. Regulation of protein-tyrosine phosphorylation and human sperm capacitation by reactive oxygen derivatives. Free Radic Biol Med 1997;22:643–56.

[41] Leclerc P, de Lamirande E, Gagnon C. Interaction between Ca2+, cyclic 3',5' adenosine monophosphate, the superoxide anion, and tyrosine phosphorylation pathways in the regulation of human sperm capacitation. J Androl 1998;19:434–43.

[42] Roy SC, Atreja SK. Tyrosine phosphorylation of a 38-kDa capacitation-associated buffalo (Bubalus bubalis) sperm protein is induced by L-arginine and regulated through a cAMP/PKA-independent pathway. Int J Androl 2008;31:12–24. doi:10.1111/j.1365-2605.2007.00745.x.

[43] O'Flaherty C, Rodriguez P, Srivastava S. L-arginine promotes capacitation and acrosome reaction in cryopreserved bovine spermatozoa. Biochim Biophys Acta 2004;1674:215–21. doi:10.1016/j.bbagen.2004.06.020.

[44] de Andrade AFC, Arruda RP, Torres MA, Pieri NCG, Leite TG, Celeghini ECC, et al. Nitric oxide in frozen-thawed equine sperm: Effects on motility, membrane integrity and sperm capacitation. Anim Reprod Sci 2018;195:176–84. doi:10.1016/j.anireprosci.2018.05.022.

[45] Misko TP, Moore WM, Kasten TP, Nickols GA, Corbett JA, Tilton RG, et al. Selective inhibition of the inducible nitric oxide synthase by aminoguanidine. Eur J Pharmacol 1993;233:119–25.

[46] Munuce MJ, Caille AM, Botti G, Berta CL. Modulation of human sperm function by follicular fluid. Andrologia 2004;36:395–401. doi:10.1111/j.1439-0272.2004.00638.x.

[47] Gye MC. Changes in sperm phosphotyrosine proteins by human follicular fluid in mice. Arch Androl 2003;49:417–22.

[48] Fàbrega A, Puigmulé M, Yeste M, Casas I, Bonet S, Pinart E. Impact of epididymal maturation, ejaculation and in vitro capacitation on tyrosine phosphorylation patterns exhibited of boar (Sus domesticus) spermatozoa. Theriogenology 2011;76:1356–66.

[49] Benoff S. Carbohydrates and fertilization: an overview. Mol Hum Reprod 1997;3:599–637. doi:10.1093/molehr/3.7.599.

[50] Yao YQ, Chiu CN, Ip SM, Ho PC, Yeung WS. Glycoproteins present in human follicular fluid that inhibit the zona- binding capacity of spermatozoa. Hum Reprod 1998;13:2541–7. doi:10.1093/humrep/13.9.2541.

[51] Chiu PCN, Koistinen R, Koistinen H, Seppala M, Lee KF, Yeung WSB. Zona-binding inhibitory factor-1 from human follicular fluid is an isoform of glycodelin. Biol Reprod 2003;69:365–72. doi:10.1095/biolreprod.102.012658.

[52] Chiu PCN, Tsang HY, Koistinen R, Koistinen H, Seppala M, Lee KF, et al. The contribution of D-mannose, L-fucose, N-acetylglucosamine, and selectin residues on the binding of glycodelin isoforms to human spermatozoa. Biol Reprod 2004;70:1710–9. doi:10.1095/biolreprod.103.023259.

[53] Salmon NA, Reijo Pera RA, Xu EY. A gene trap knockout of the abundant sperm tail protein, outer dense fiber 2, results in preimplantation lethality. Genesis 2006;44:515–22. doi:10.1002/dvg.20241.

[54] Nixon B, Bromfield EG, Cui J, De Iuliis GN. Heat Shock Protein A2 (HSPA2): Regulatory Roles in Germ Cell Development and Sperm Function. Adv. Anat. Embryol. Cell Biol., vol. 222, 2017, p. 67–93. doi:10.1007/978-3-319-51409-3_4.

[55] Bornstein C, Brosh R, Molchadsky A, Madar S, Kogan-Sakin I, Goldstein I, et al. SPATA18, a Spermatogenesis-Associated Gene, Is a Novel Transcriptional Target of p53 and p63. Mol Cell Biol 2011;31:1679–89. doi:10.1128/MCB.01072-10.

[56] Dix DJ, Rosario-Herrle M, Gotoh H, Mori C, Goulding EH, Barrett C V, et al. Developmentally regulated expression of Hsp70-2 and a Hsp70-2/lacZ transgene during spermatogenesis. Dev Biol 1996;174:310–21. doi:10.1006/dbio.1996.0076.

[57] Rosario MO, Perkins SL, O'Brien DA, Allen RL, Eddy EM. Identification of the gene for the developmentally expressed 70 kDa heat-shock protein (P70) of mouse spermatogenic cells. Dev Biol 1992;150:1–11.

[58] Mori C, Nakamura N, Dix DJ, Fujioka M, Nakagawa S, Shiota K, et al. Morphological analysis of germ cell apoptosis during postnatal testis development in normal and Hsp 70-2 knockout mice. Dev Dyn 1997;208:125–36.

[59] Redgrove KA, Anderson AL, Dun MD, McLaughlin EA, O'Bryan MK, Aitken RJ, et al. Involvement of multimeric protein complexes in mediating the capacitation-dependent binding of

human spermatozoa to homologous zonae pellucidae. Dev Biol 2011;356:460–74. doi:10.1016/j.ydbio.2011.05.674.

[60] Souès S, Kann M-L, Fouquet J-P, Melki R. The cytosolic chaperonin CCT associates to cytoplasmic microtubular structures during mammalian spermiogenesis and to heterochromatin in germline and somatic cells. Exp Cell Res 2003;288:363–73.

[61] Ferramosca A, Zara V. Bioenergetics of Mammalian Sperm Capacitation. Biomed Res Int 2014;2014:1–8. doi:10.1155/2014/902953.

[62] du Plessis SS, Agarwal A, Mohanty G, van der Linde M. Oxidative phosphorylation versus glycolysis: what fuel do spermatozoa use? Asian J Androl 2015;17:230–5. doi:10.4103/1008-682X.135123.

[63] Storey BT. Mammalian sperm metabolism: oxygen and sugar, friend and foe. Int J Dev Biol 2008;52:427–37. doi:10.1387/ijdb.072522bs.

[64] Ji Z-L, Duan Y-G, Mou L-S, Allam J-P, Haidl G, Cai Z-M. Association of heat shock proteins, heat shock factors and male infertility. Asian Pacific J Reprod 2012;1:76–84. doi:10.1016/S2305-0500(13)60053-6.

[65] Vijayaraghavan S, Goueli SA, Davey MP, Carr DW. Protein kinase A-anchoring inhibitor peptides arrest mammalian sperm motility. J Biol Chem 1997;272:4747–52. doi:10.1074/jbc.272.8.4747.

[66] Brohmann H, Pinnecke S, Hoyer-Fender S. Identification and Characterization of New cDNAs Encoding Outer Dense Fiber Proteins of Rat Sperm. J Biol Chem 1997;272:10327–32. doi:10.1074/jbc.272.15.10327.

[67] Petersen C, Füzesi L, Hoyer-Fender S. Outer dense fibre proteins from human sperm tail: molecular cloning and expression analyses of two cDNA transcripts encoding proteins of 70 kDa. Mol Hum Reprod 1999;5:627–35. doi:10.1093/molehr/5.7.627.

[68] Hashemitabar M, Sabbagh S, Orazizadeh M, Ghadiri A, Bahmanzadeh M. A proteomic analysis on human sperm tail: comparison between normozoospermia and asthenozoospermia. J Assist Reprod Genet 2015;32:853–63. doi:10.1007/s10815-015-0465-7.

[69] Chemes HE, Olmedo SB, Carrere C, Oses R, Carizza C, Leisner M, et al. Ultrastructural pathology of the sperm flagellum: association between flagellar pathology and fertility prognosis in severely asthenozoospermic men. Hum Reprod 1998;13:2521–6. doi:10.1093/humrep/13.9.2521.

[70] Brener E, Rubinstein S, Cohen G, Shternall K, Rivlin J, Breitbart H. Remodeling of the Actin Cytoskeleton During Mammalian Sperm Capacitation and Acrosome Reaction1. Biol Reprod 2003;68:837–45. doi:10.1095/biolreprod.102.009233.

[71] Finkelstein M, Megnagi B, Ickowicz D, Breitbart H. Regulation of sperm motility by PIP2(4,5) and actin polymerization. Dev Biol 2013;381:62–72. doi:10.1016/j.ydbio.2013.06.014.

[72] Breitbart H, Finkelstein M. Actin cytoskeleton and sperm function. Biochem Biophys Res Commun 2018;506:372–7. doi:10.1016/j.bbrc.2017.11.001.

[73] Buzadzic B, Vucetic M, Jankovic A, Stancic A, Korac A, Korac B, et al. New insights into male (in)fertility: the importance of NO. Br J Pharmacol 2015;172:1455–67. doi:10.1111/bph.12675.

[74] Amaral A, Castillo J, Ramalho-Santos J, Oliva R. The combined human sperm proteome: cellular pathways and implications for basic and clinical science. Hum Reprod Update 2014;20:40–62. doi:10.1093/humupd/dmt046.

[75] Hou C-C, Yang W-X. New insights to the ubiquitin-proteasome pathway (UPP) mechanism during spermatogenesis. Mol Biol Rep 2013;40:3213–30. doi:10.1007/s11033-012-2397-y.

[76] Sutovsky P, Moreno R, Ramalho-Santos J, Dominko T, Thompson WE, Schatten G. A putative, ubiquitin-dependent mechanism for the recognition and elimination of defective spermatozoa in the mammalian epididymis. J Cell Sci 2001;114:1665–75.

[77] Baska KM, Manandhar G, Feng D, Agca Y, Tengowski MW, Sutovsky M, et al. Mechanism of extracellular ubiquitination in the mammalian epididymis. J Cell Physiol 2008;215:684–96. doi:10.1002/jcp.21349.

[78] Sutovsky P, Moreno RD, Ramalho-Santos J, Dominko T, Simerly C, Schatten G. Ubiquitin tag for sperm mitochondria. Nature 1999;402:371–2. doi:10.1038/46466.

[79] Heberle H, Meirelles GV, da Silva FR, Telles GP, Minghim R. InteractiVenn: a web-based tool for the analysis of sets through Venn diagrams. BMC Bioinformatics 2015;16:169. doi:10.1186/s12859-015-0611-3.

CHAPTER 3

Follicular fluid nitrite and nitrate levels in oocyte donors: correlation with ovarian response

ABSTRACT

NO, a key regulatory molecule in the FF, has been investigated as a possible biomarker to predict ovarian response in stimulated cycles. Nevertheless, a consensus on whether or not NO can help in this context has not been reached. We simultaneously measured the oxidation products of NO, NO_2 and NO_3, via HPLC-UV/VIS in FF samples from seventy-two oocyte donors. We found no associations of FF NO_2, NO_3, total NO or NO_3/NO_2 ratio with total or MII oocyte yield. However, NO_2 and NO_3 levels were related to the yield of MII oocytes when this outcome was expressed as a proportion of all oocytes retrieved. The adjusted MII proportion in the lowest and highest NO_2 levels were 68% (58-77%) and 79% (70-85%), respectively (P, linear trend=0.02); whereas the adjusted MII proportion in extreme tertiles of NO_3 levels were 79% (70-85%) and 68% (57-77%) (P, linear trend=0.03). These results suggest that the FF concentrations of NO_2 and NO_3 may be a useful tool in predicting how healthy oocyte donors respond to superovulation.

1. INTRODUCTION

A large number of couples of reproductive age struggle with infertility issues, the causes of which are not always clear [1]. For this reason, several studies tried to identify new biochemical markers that can affect gamete and embryo quality and may predict the outcome of infertility treatment with IVF [2].

NO has emerged as a candidate predictor of ovarian response and IVF outcomes [3]. Apart from being a well-known regulator of vasodilation and neurotransmission [4], NO has also been linked to the granulosa cell function [5], meiotic resumption [6] and prevention of oocyte aging [7], as well as ovulation [8]. However, when investigating the relation of intrafollicular levels of NO with oocyte quality, fertilization potential, embryo quality, implantation and pregnancy rates in patients undergoing IVF, the results are contradictory. On one hand, evidence suggests that NO levels in the FF are inversely associated with the fertilization of mature oocytes and the ability of the subsequent embryo to cleave normally [9]. Furthermore, a negative correlation between FF NO levels and embryo morphology was identified [3,10]. On the other hand, other studies found no differences in relation to oocyte and embryo quality [2,11]. The relationship between FF NO and pregnancy outcome is also uncertain [2,11,12].

The controversy between these data might reside in the unstable nature of NO [13]. NO has a short biological half-life of 5 s or less [14], which makes its direct measurement difficult. However, NO is oxidized in blood and tissues leading to the formation of two stable end-products, NO_2 and NO_3 [15]. The methods for NO_2 and NO_3 detection have limitations such as the time required for the analysis, interference from other ions or the difficulty to detect NO_2 and NO_3 at the same time and in minor concentrations [16]. The simultaneous detection of low NO_2 and NO_3 concentrations has been described in mammal blood, urine and in plant samples by HPLC-UV/VIS [16]. This study aims, first of all, to apply this method with some minor modifications to determine the FF levels of NO_2, NO_3, total NO, NO_3/NO_2 ratio in oocyte donors. Subsequently, it aims to further clarify any associations between these parameters and ovarian response in healthy women undergoing ovarian stimulation as part of their participation in an oocyte donation program.

2. MATERIALS AND METHODS

Ethics

This study was approved by the Ethics Review Committee of CEIC Hospital General Universitario Jose Maria Morales Meseguer (Murcia, Spain) (Approval No. EST: 06/17). Women

participating in the gamete donation program at IVI-RMA Global (Murcia, Spain) were invited to participate in the study. All the donors who accepted provided their written informed consent.

Clinical and lifestyle-related data

The following data regarding the donation cycle were collected from IVI-RMA Global (Murcia, Spain) for all participants: total and MII oocyte yield, number of stimulation days, 17β-estradiol (E2) peak, total dose of FSH and oocyte fate (fresh transfer, vitrified and mixed). Moreover, the donors completed a questionnaire reporting information about their age, body mass index (BMI), sleep time, diet, coffee and alcohol consumption, smoking history, leisure physical activity and sedentary behavior.

FF samples

Superovulation was achieved by means of a human recombinant follicle-stimulating hormone and a gonadotrophin-releasing hormone antagonist, as previously described [17]. When the follicles reached an average diameter of 17.5-18 mm, a GnRH agonist was administered to induce ovulation [17]. Approximately thirty-six hours later, dominant follicles were punctured transvaginally under ultrasound guidance, and FF was aspirated together with the oocyte. After oocyte retrieval, the FF samples were centrifuged during 15 min at 1500 × g. The supernatant was filtered using 0.22 µm filter units (Merck KGaA, Darmstadt, Germany) to remove cellular debris, then aliquoted and stored at -20 °C until use. Before and after centrifugation, an aliquot from 26 FF samples was used to determine Hb levels with a HemoCue Plasma/Low Hb System (Ängelholm, Sweden). This was done to ensure that the puncture procedure did not affect the Hb levels in the fluid, which, if present after the FF has been processed, can cause NO_2 reduction or oxidation [15].

NO_2 and NO_3 measurements in FF

NO_2 and NO_3 levels in FF samples were measured by HPLC-UV/VIS, using a method previously described by Croitoru MD [16] with some minor changes. The analysis was carried out on an Agilent 1100 Series HPLC System (Agilent Technologies, Santa Clara, CA, USA) equipped with a thermostated microwell plate autosampler, a quaternary pump, and a multiple wavelength absorbance detector. Standards and samples (40 µL) were injected into an Agilent Zorbax Eclipse XDB-C18 HPLC column (4.6 × 150 mm, 5 µm), thermostated at 25 °C, and eluted at a flow rate of 400 µL/min during the whole separation. Mobile phase A consisted of 5 mM tetrabutylammonium hydroxide pH 2.5 (Sigma-Aldrich Química S.A., Madrid, Spain) and 8% *v/v* acetonitrile (VWR Chemicals, Barcelona, Spain) in water, while mobile phase B was methanol (VWR Chemicals, Barcelona, Spain). The gradient elution program was: 100% solvent A for 10 min; a linear gradient from 0 to 50% solvent

B for 20 min; 10 min at constant 100% solvent B. The column was equilibrated with the starting composition of the mobile phase for 15 min before each analytical run. The 206 nm absorbance signal was recorded.

HPLC standards were prepared in Milli-Q water using reagent grade sodium nitrite and sodium nitrate (Sigma-Aldrich Química S.A., Madrid, Spain). Both standards were prepared at a concentration of 1 mM and serial dilutions from 100 to 0.1 μM were used to obtain the calibration curve for the analysis. FF samples were thawed and filtered through Amicon 3K centrifugal units (Merck KGaA, Darmstadt, Germany) to eliminate proteins. The centrifugal units were first rinsed with Milli-Q water to equilibrate the membrane and then centrifuged for 10 min at 14000 × g. The receiver tube was replaced with a new one and 400 μL of sample were added to the centrifugal unit and centrifuged for 60 min at 14000 × g. The clean filtrates were used for the analysis. The UV chromatograms at 206 nm from both standards and samples were analyzed with Chemstation Rev B.01.03.SR2 (Agilent Technologies, Santa Clara, CA, USA). The NO_2 and NO_3 peak areas in the standard solutions were used for the calculation of the calibration curve, from which the concentration in samples was obtained. The measurements were performed in duplicate and the sum of mean values of NO_2 and NO_3 levels were used to calculate total NO concentration [18]. The ratio between mean values of NO_3 and NO_2 levels was also determined.

Statistical analysis

Descriptive statistics were calculated for demographic, lifestyle and first donation cycle characteristics in the entire cohort by tertiles of NO_2, NO_3, total NO and NO_3/NO_2 ratio. The presence of any associations was evaluated by using ANOVA and chi-square tests for continuous and categorical variables, respectively. These data were presented as mean (standard deviation) or number of women (%).

Multivariable mixed Poisson and logistic regression models with random slopes to account for repeated observations within woman were used to compare total and MII oocyte yields, as well as the proportion of MII oocytes, across tertiles of NO_2, NO_3, total NO and NO_3/NO_2 ratio. Categorical covariables were included using indicators for missing covariates when necessary. Multivariable-adjusted models included terms for age, body mass index, sleep time, coffee intake, smoking history and leisure physical activity, as potential confounders of the relation between NO metabolites and measures of ovarian response to hyperstimulation. Tests for linear trend were conducted by modeling the tertiles of each metabolite, using the median analyte concentration values in each tertile, as a continuous linear term. All analyses were performed in SAS 9.4 (SAS Institute).

3. RESULTS

Seventy-two women participating in the oocyte donation program at IVI-RMA Global Murcia (Spain), between February 2017 and September 2018, were included in our cohort. FF was obtained at oocyte retrieval in 93 oocyte donation cycles to measure NO_2 and NO_3 levels by HPLC-UV/VIS (Fig. 1). The first peak, located at 10.2 min was identified as NO_2, while the peak at 31.6 min was identified as NO_3. When analyzing the chromatogram from different FF samples, we observed the same peaks with the same retention times (Fig. 1).

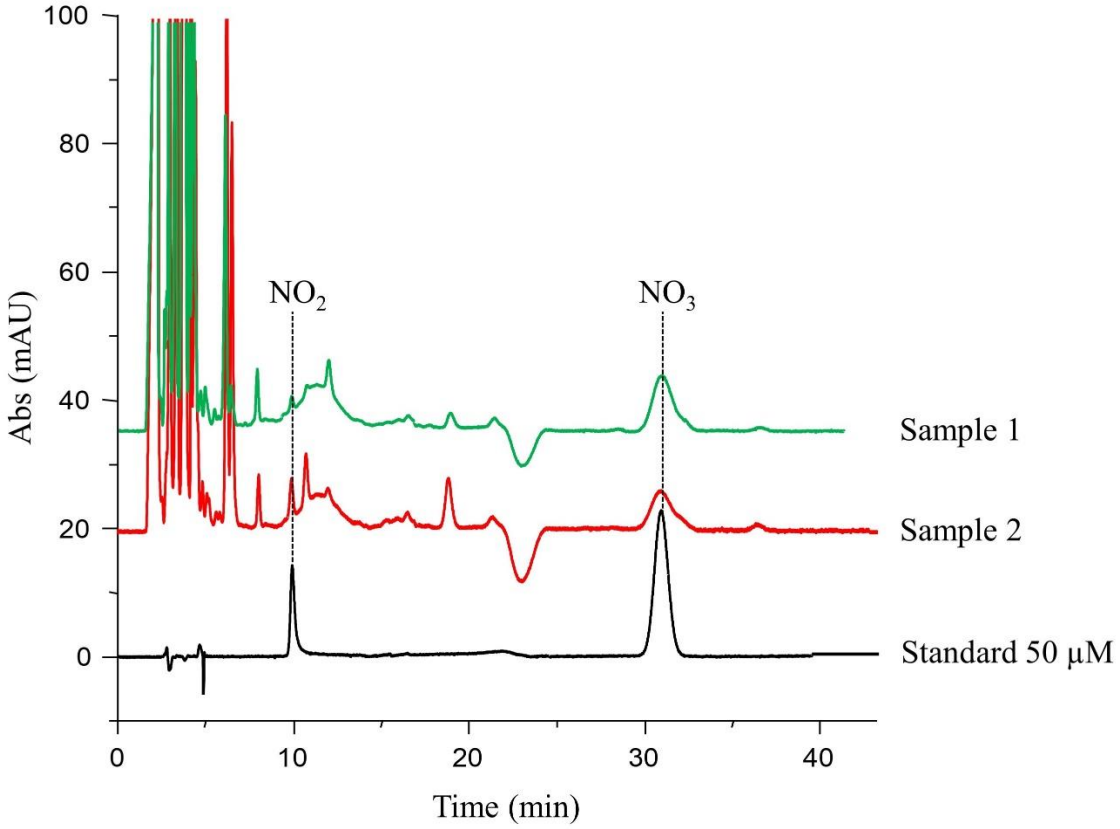


Figure 1. HPLC-UV/VIS chromatogram. The peak located at 10.2 min was identified as NO_2, while the peak at 31.6 min was identified as NO_3. The peak areas of each compound in the standard solutions were used for the calculation of the calibration curve, from which the concentrations in follicular fluid samples (e.g. Sample 1 and 2) were obtained.

The FF concentrations of NO_2, NO_3, total NO and the NO_3/NO_2 ratio are reported in Table 1. NO_2 levels ranged from 0.7 to 96.1 µM, NO_3 levels ranged from 4.9 to 39.7 µM, total NO levels ranged from 5.6 to 109.5 µM and NO_3/NO_2 ratio ranged from 0.1 to 31.5. NO_2 and NO_3 concentrations were unrelated to each other (r=-0.01). NO_2 was positively correlated with total NO and negatively correlated with the NO_3/NO_2 ratio (Table 1).

No significant differences were found when analyzing the following variables across tertiles of NO_2 and NO_3: age at egg donation, BMI, Mediterranean diet score, coffee and occasional alcohol intake, second hand exposure to smoke and sedentary behavior (Table 2). On the other hand, women with higher FF NO_2 levels were more likely to sleep less [mean (SD) of 7.0 (2.0) h/day] and spend more time per week in leisure activities [mean (SD) of 3.7 (6.0) h/week]. Moreover, higher NO_2 and NO_3 concentrations were present in donors who smoked (22.2 and 23.6%, respectively), either at the time of the study or in the past. The characteristics of the first donation cycle, such as the number of stimulation days, total dose of FSH and the oocyte fate, were similar across tertiles of NO_2 and NO_3, but donors with low FF NO_2 levels likely had a higher E2 peak [mean (SD) of 2004.2 (1140.8) mg/dL].

FF NO_2 and NO_3 were unrelated to total or mature oocyte yield (Table 3). The multivariable-adjusted MII yield (95% CI) for women in the lowest and highest tertiles of NO_2 was 12.4 (10.2, 15.1) and 13.2 (10.9, 16.0) (P, linear trend=0.38) and 14.1 (11.7, 17.1) and 12.2 (9.9, 15.0) for NO_3 (P, linear trend=0.14), respectively. When MII oocytes were considered as the proportion of total oocytes, however, the proportion of MII oocytes increased with increasing FF NO_2 levels but decreased with increasing NO_3 levels. The adjusted proportion (95% CI) of MII oocytes for women in the lowest and highest FF levels of NO_2 were 68% (58-77%) and 79% (70-85%) (P, linear trend=0.02); whereas the proportion of MII oocytes for women in extreme tertiles of FF NO_3 levels were 79% (70-85%) and 68% (57-77%) (P, linear trend=0.03). Total NO and the NO_3/NO_2 ratio were unrelated to the total and mature oocyte yield, whether expressed in absolute or relative terms (Table 3).

Table 1. Follicular fluid levels of NO_2, NO_3, total NO and NO_3/NO_2 ratio and Pearson correlation coefficients between these parameters.

				r			
	MIN	**MAX**	**MEAN (SD)**	**NO_2**	**NO_3**	**TOTAL NO**	**NO_3/NO_2 RATIO**
NO_2	0.7	96.1	14.7 (12.3)	1.00	-0.01	0.89	-0.40
NO_3	4.9	39.7	17.3 (6.3)		1.00	0.45	0.14
TOTAL NO	5.6	109.5	31.9 (13.7)			1.00	-0.29
NO_3/NO_2 RATIO	0.1	31.5	2.6 (4.4)				1.00

Minimum, maximum and mean (standard deviation) concentrations of NO_2, NO_3 and total NO are expressed as μM. The values are representative of 93 follicular fluid samples.

Table 2. Demographic, lifestyle and first cycle characteristics by NO_2 and NO_3 tertiles.

	NO_2				NO_3			
	1st tertile, N=25	**2nd tertile, N=23**	**3rd tertile, N=24**	**p-value[2]**	**1st tertile, N=24**	**2nd tertile, N=24**	**3rd tertile, N=24**	**p-value[2]**
Demographic and lifestyle characteristics[1]								
Age at egg donation, years	24.9 (4.9)	23.7 (3.9)	24.6 (4.0)	0.83	23.6 (4.2)	24.6 (4.2)	25.1 (4.4)	0.22
Body Mass Index, kg/m^2	22.4 (3.1)	22.7 (2.6)	23.2 (3.3)	0.35	23.6 (3.1)	22.3 (2.9)	22.3 (2.9)	0.12
Sleep time/day, h	8.7 (1.9)	8.6 (1.1)	7.0 (2.0)	0.01	7.6 (2.5)	8.1 (2.1)	8.2 (1.2)	0.45
Mediterranean diet score [33]	34.1 (6.1)	31.6 (6.8)	32.6 (7.5)	0.53	31.2 (7.6)	35.7 (5.6)	32.0 (6.5)	0.80
Coffee intake, servings/w	4.2 (10.1)	2.2 (3.0)	8.2 (9.0)	0.15	4.7 (8.5)	3.8 (6.2)	6.7 (10.1)	0.46
Occasional alcohol intake, N (%)	14 (19.4)	12 (16.7)	12 (16.7)	0.91	11 (15.3)	13 (18.1)	14 (19.4)	0.68
Ever smoker, N (%)	15 (20.8)	5 (6.9)	16 (22.2)	0.00	11 (15.3)	8 (11.1)	17 (23.6)	0.03
Self-reported exposure to second hand smoke, N (%)	6 (11.3)	6 (11.3)	6 (11.3)	0.93	8 (15.1)	4 (7.6)	6 (11.3)	0.37
Leisure moderate/vigorous activity, h/w	0.7 (1.8)	1.4 (2.8)	3.7 (6.0)	0.04	2.4 (2.9)	1.2 (2.6)	2.2 (5.8)	0.96
Sedentary behavior, h/w	25.4 (28.4)	23.2 (11.6)	30.3 (21.6)	0.52	28.0 (21.2)	23.2 (28.0)	28.0 (16.4)	0.95
First cycle characteristics[1]								
Number of stimulation days, N	9.9 (1.8)	9.8 (1.1)	9.7 (1.1)	0.76	9.7 (1.2)	9.5 (1.0)	10.2 (1.8)	0.32
E2 peak, mg/dL	2004.2 (1140.8)	1474.7 (668.5)	1328.1 (667.1)	0.02	1619.4 (726.6)	1748.6 (1275.1)	1535.3 (678.1)	0.78
FSH total dose, IU/L	1450.5 (395.0)	1413.3 (531.0)	1649.6 (453.1)	0.19	1398.1 (570.7)	1516.5 (352.3)	1604.2 (429.5)	0.19
Oocyte fate, N (%)				0.65				0.58
▪ Fresh transfer	12 (22.6)	6 (11.3)	7 (13.2)		7 (13.2)	8 (15.1)	10 (18.9)	
▪ Vitrified	3 (5.7)	1 (1.9)	3 (5.7)		4 (7.6)	2 (3.8)	1 (1.9)	
▪ Mixed	6 (11.3)	7 (13.2)	8 (15.1)		6 (11.3)	8 (15.1)	7 (13.2)	

[1]Data are presented as mean (standard deviation) or number of women (%).

[2]Differences across categories were tested using an ANOVA test for continuous variables and a Chi Square test for categorical variables.

Table 3. Association between NO-related parameters and the adjusted oocyte yield, number and proportion of MII oocytes in women donors.

	N cycles / N donors	Oocyte yield, N[1]	MII oocytes, N[1]	MII oocyte proportion (%)[2]
NO_2	93 / 72			
1st tertile	31 / 25	18.2 (15.0, 22.1)	12.4 (10.2, 15.1)	68 (58, 77)
2nd tertile	31 / 23	18.5 (15.1, 22.6)	14.0 (11.4, 17.1)	78 (69, 85)
3rd tertile	31 / 24	18.3 (15.1, 22.1)	13.2 (10.9, 16.0)	79 (70, 85)
P, linear trend[3]		0.94	0.38	0.02
NO_3	93 / 72			
1st tertile	30 / 24	18.5 (15.4, 22.4)	14.1 (11.7, 17.1)	79 (70, 85)
2nd tertile	32 / 24	18.1 (15.0, 21.9)	13.2 (10.9, 16.1)	78 (70, 85)
3rd tertile	31 / 24	18.3 (15.0, 22.5)	12.2 (9.9, 15.0)	68 (57, 77)
P, linear trend[3]		0.88	0.14	0.03
Total NO	93 / 72			
1st tertile	30 / 26	19.2 (16.0, 23.1)	13.7 (11.4, 16.5)	74 (65, 81)
2nd tertile	31 / 20	21.2 (17.3, 25.8)	15.2 (12.4, 18.6)	77 (68, 84)
3rd tertile	32 / 26	16.4 (13.7, 19.7)	12.2 (10.1, 14.6)	77 (69, 84)
P, linear trend[3]		0.14	0.31	0.41
NO_3/NO_2 ratio	93 / 72			
1st tertile	31 / 23	21.7 (16.7, 28.1)	14.1 (10.7, 18.6)	68 (52, 80)
2nd tertile	31 / 23	16.8 (13.8, 20.6)	12.9 (10.4, 15.9)	82 (73, 88)
3rd tertile	31 / 26	17.2 (13.4, 22.2)	12.6 (9.6, 16.5)	74 (61, 84)
P, linear trend[3]		0.18	0.54	0.42

Models were run using [1]Poisson regression with log link and [2]binomial regression with logit link. All data are presented as least square means (95% CI), adjusted for age, body mass index, sleep time, coffee intake, smoking history and leisure physical activity.

[3]P, linear trend was calculated by modeling the tertiles of each metabolite, using the median analyte concentration values in each tertile as a continuous linear term.

4. DISCUSSION

One of the factors associated with successful pregnancy during IVF is oocyte quality [11]. The oocyte development takes place in a dynamic microenvironment, where the complex composition of the FF is very important [19]. Among other molecules, FF contains free radicals, like NO, which is actively synthesized by the granulosa cells [11]. This means that NO and/or its by-products may be potential biomarkers for IVF outcome and several studies tested this hypothesis in patients undergoing fertility treatment [3,9–11,19–21].

The assessment of NO concentration can be performed indirectly by assaying two anions, i.e. NO_2 and NO_3 [22]. In the present work, we described how a previously validated technique for measuring simultaneously these ions [16], can also be applied in FF samples.

The levels of NO_2 and NO_3, described in our cohort, did not predict the total number of oocytes recovered from the donors, nor the MII oocytes count. Other studies described similar findings. Yalçınkaya *et al*. [2] reported the absence of a correlation when evaluating the relationship between NO and IVF parameters such as the number of mature oocytes, fertilization rate and embryo grading. Lee *et al*. [11] found no significant differences between FF NO levels and the maturity and quality of the oocyte. Moreover, the NO_2/NO_3 concentrations measured in both serum and FF were not good markers of ovarian response or pregnancy in IVF cycles [13].

Interestingly, our data suggests that the FF concentrations of NO_2 and NO_3 are associated with the proportion of MII oocytes, particularly, the latter was related directly to NO_2 levels and inversely to NO_3 levels. Even though these stable ions derive from NO, previous research reports evidenced that they can be physiologically recycled to form again NO [15], likely to be employed for protein S-nitrosylation during maturation process of the oocyte [6]. According to our results, FF NO_2 levels appear to be more representative for NO formation, as shown by the Pearson correlation coefficients. The formation of NO_2 takes place, for instance, by NO auto-oxidation, NO_3 reduction [15], or through a reaction catalyzed by the multi-copper oxidase ceruloplasmin [23], which is also a FF component [24–26]. It has been shown that its levels depend on the ovarian stimulation protocol [24] and it was described as an indicator of oocyte maturation, since the ceruloplasmin concentration was higher in follicles containing a mature egg [27] and that later underwent cleavage [25]. NO_2 formation via ceruloplasmin also produces nitrous acid [23]. Both these species can be converted back into NO in the presence of ascorbate (reviewed by [15]), which has been identified in the FF [28,29].

On the other hand, higher FF NO_3 levels were found to be consistent with the presence of nitrotyrosine in granulosa cells, which is indicative of peroxynitrite synthesis [20]. The synthesis of peroxynitrite takes place when NO interacts with the superoxide anion [30]. The latter species is physiologically produced during folliculogenesis, but lifestyle factors can lead to an unbalance in its regulation [31]. In turn, the peroxynitrite causes lipid peroxidation and cellular damage [32]. Goud *et al*. [20] reported increased FF NO_3 levels in women with versus women without endometriosis. Additionally, the affected women who achieved a pregnancy had significantly lower FF NO_3 levels. The authors, therefore, suggested that the presence of high concentrations of NO_3 and peroxynitrite in the oocyte microenvironment may contribute to a poor follicular health and oocyte quality. This might justify the negative correlation between the proportion of MII oocytes and the NO_3 levels reported in our study.

5. ACKNOWLEDGEMENTS

The authors involved in this study would like to thank all the staff from the Embryology laboratory at IVI-RMA Global (Murcia, Spain) for their assistance in recruiting the FF donors. Furthermore, the authors thank Prof. Joaquín Ortuño Sánchez-Pedreño and Dr. Alejandro Torrecillas for their help with the NO_2 and NO_3 measurements, as well as Prof. Javier Corral de la Calle, for providing the HemoCue Plasma/Low Hb System.

6. SUPPORTING INFORMATION

Supplementary table. Hemoglobin levels in follicular fluid samples.

FF sample	Hb before processing (g/dL)	Hb after processing (g/dL)
1	0.02 (0.00)	0.00 (0.00)
2	0.02 (0.00)	0.00 (0.00)
3	0.00 (0.00)	0.00 (0.00)
4	0.02 (0.00)	0.00 (0.00)
5	0.02 (0.00)	0.00 (0.00)
6	0.01 (0.00)	0.00 (0.00)
7	0.04 (0.00)	0.00 (0.00)
8	0.10 (0.00)	0.00 (0.00)
9	0.00 (0.00)	0.00 (0.00)
10	0.00 (0.00)	0.00 (0.00)
11	0.00 (0.00)	0.00 (0.00)
12	0.03 (0.00)	0.00 (0.00)
13	0.02 (0.00)	0.00 (0.00)
14	0.02 (0.00)	0.00 (0.00)
15	0.06 (0.00)	0.00 (0.00)
16	0.03 (0.00)	0.00 (0.00)
17	0.02 (0.00)	0.00 (0.00)
18	0.02 (0.00)	0.00 (0.00)
19	0.08 (0.00)	0.00 (0.00)
20	0.00 (0.00)	0.00 (0.00)
21	0.02 (0.00)	0.00 (0.00)
22	0.00 (0.00)	0.00 (0.00)
23	0.05 (0.00)	0.00 (0.00)
24	0.00 (0.00)	0.00 (0.00)
25	0.04 (0.00)	0.00 (0.00)
26	0.00 (0.00)	0.00 (0.00)

FF, follicular fluid; Hb, hemoglobin; measurements were performed in duplicate and results are represented as mean (SD).

Supplementary table. Demographic, lifestyle and first cycle characteristics by total NO tertiles.

	TOTAL NO			
	1st tertile, N=26	**2nd tertile, N=20**	**3rd tertile, N=26**	**p-value[2]**
Demographic and lifestyle characteristics[1]				
Age at egg donation, years	24.2 (4.2)	24.6 (5.2)	24.5 (3.7)	0.80
Body Mass Index, kg/m^2	22.7 (3.0)	22.5 (2.6)	22.9 (3.3)	0.86
Sleep time/day, h	8.7 (2.0)	8.0 (1.2)	7.4 (2.1)	0.07
Mediterranean diet score [33]	33.1 (7.6)	34.4 (4.6)	31.8 (7.3)	0.55
Coffee intake, servings/w	4.0 (10.1)	3.5 (5.2)	7.1 (8.5)	0.26
Occasional alcohol intake, N (%)	12 (16.7)	14 (19.4)	12 (16.7)	0.19
Ever smoker, N (%)	11 (15.3)	8 (11.1)	17 (23.6)	0.14
Self-reported exposure to second hand smoke, N (%)	8 (15.1)	4 (7.6)	6 (11.3)	0.77
Leisure moderate/vigorous activity, h/w	1.8 (2.9)	0.7 (2.3)	3.0 (5.8)	0.42
Sedentary behavior, h/w	24.2 (26.4)	23.1 (15.4)	31.0 (20.6)	0.37
First cycle characteristics[1]				
Number of stimulation days, N	9.7 (1.1)	10.1 (2.0)	9.7 (1.2)	0.98
E2 peak, mg/dL	1983.2 (1149.2)	1465.4 (780.3)	1392.6 (639.9)	0.04
FSH total dose, IU/L	1474.3 (488.5)	1477.5 (339.0)	1566.7 (511.1)	0.54
Oocyte fate, N (%)				0.64
▪ Fresh transfer	10 (18.9)	6 (11.3)	9 (17.0)	
▪ Vitrified	4 (7.6)	2 (3.8)	1 (1.9)	
▪ Mixed	6 (11.3)	6 (11.3)	9 (17.0)	

[1]Data are presented as mean (standard deviation) or number of women (%).

[2]Differences across categories were tested using an ANOVA test for continuous variables and a Chi Square test for categorical variables.

Supplementary table. Demographic, lifestyle and first cycle characteristics by tertiles of NO_3/NO_2 ratio.

	NO_3/NO_2 RATIO			
	1st tertile, N=23	**2nd tertile, N=23**	**3rd tertile, N=26**	**p-value[2]**
Demographic and lifestyle characteristics[1]				
Age at egg donation, years	23.7 (4.1)	24.0 (4.0)	25.5 (4.6)	0.15
Body Mass Index, kg/m^2	23.4 (2.9)	22.7 (3.0)	22.2 (3.0)	0.17
Sleep time/day, h	7.2 (2.2)	7.9 (1.6)	8.7 (1.8)	0.04
Mediterranean diet score [33]	31.3 (8.6)	34.2 (4.7)	33.2 (6.4)	0.46
Coffee intake, servings/w	6.7 (8.8)	3.9 (6.3)	5.0 (9.7)	0.58
Occasional alcohol intake, N (%)	11 (15.3)	11 (15.3)	16 (22.2)	0.53
Ever smoker, N (%)	13 (18.1)	8 (11.1)	15 (20.8)	0.21
Self-reported exposure to second hand smoke, N (%)	6 (11.3)	5 (9.4)	7 (13.2)	0.96
Leisure moderate/vigorous activity, h/w	2.5 (3.0)	3.2 (7.0)	0.8 (1.9)	0.21
Sedentary behavior, h/w	27.5 (21.4)	24.4 (15.0)	27.1 (25.8)	0.98
First cycle characteristics[1]				
Number of stimulation days, N	9.8 (1.2)	9.5 (0.8)	10.0 (1.8)	0.62
E2 peak, mg/dL	1465.1 (525.3)	1299.3 (745.1)	1986.7 (1145.1)	0.06
FSH total dose, IU/L	1543.3 (570.5)	1622.4 (333.8)	1405.0 (433.2)	0.31
Oocyte fate, N (%)				0.85
▪ Fresh transfer	6 (11.3)	7 (13.2)	12 (22.7)	
▪ Vitrified	3 (5.7)	2 (3.8)	2 (3.8)	
▪ Mixed	7 (13.2)	6 (11.3)	8 (15.1)	

[1]Data are presented as mean (standard deviation) or number of women (%).

[2]Differences across categories were tested using an ANOVA test for continuous variables and a Chi Square test for categorical variables.

Supplementary table. Association between NO-related parameters and the unadjusted oocyte yield, number and proportion of MII oocytes in women donors.

	N cycles / N donors	Oocyte yield, N[1]	MII oocytes, N[1]	MII oocyte proportion (%)[2]
NO_2	93 / 72			
1st tertile	31 / 25	18.2 (15.8, 20.9)	12.8 (11.0, 14.8)	71 (64, 77)
2nd tertile	31 / 23	17.9 (15.5, 20.6)	14.3 (12.3, 16.5)	81 (75, 86)
3rd tertile	31 / 24	19.1 (16.6, 22.0)	14.0 (12.0, 16.2)	76 (70, 82)
P, linear trend[3]		0.62	0.30	0.08
NO_3	93 / 72			
1st tertile	30 / 24	18.3 (15.8, 21.1)	14.4 (12.4, 16.8)	80 (74, 85)
2nd tertile	32 / 24	18.2 (15.9, 20.8)	14.0 (12.2, 16.1)	80 (74, 84)
3rd tertile	31 / 24	18.7 (16.2, 21.7)	12.7 (10.9, 14.8)	69 (62, 76)
P, linear trend[3]		0.80	0.21	0.02
Total NO	93 / 72			
1st tertile	30 / 26	18.3 (15.9, 21.1)	13.5 (11.6, 15.6)	75 (68, 81)
2nd tertile	31 / 20	20.4 (17.7, 23.6)	15.3 (13.2, 17.8)	78 (72, 84)
3rd tertile	32 / 26	16.7 (14.5, 19.2)	12.5 (10.8, 14.5)	76 (69, 82)
P, linear trend[3]		0.29	0.44	0.76
NO_3/NO_2 ratio	93 / 72			
1st tertile	31 / 23	20.1 (17.5, 23.0)	14.8 (12.8, 17.0)	76 (69, 82)
2nd tertile	31 / 23	16.9 (14.7, 19.4)	13.5 (11.7, 15.6)	83 (77, 87)
3rd tertile	31 / 26	18.4 (16.0, 21.1)	12.8 (11.0, 14.8)	69 (62, 76)
P, linear trend[3]		0.37	0.12	0.05

Models were run using [1]Poisson regression with log link and [2]binomial regression with logit link. All data are presented as not-adjusted least square means (95% CI).

[3]P, linear trend was calculated by modeling the tertiles of each metabolite, using the median analyte concentration values in each tertile as a continuous linear term.

7. REFERENCES

[1] Agarwal A, Allamaneni SSR. Role of free radicals in female reproductive diseases and assisted reproduction. Reprod Biomed Online 2004;9:338–47.

[2] Yalçınkaya E, Cakıroğlu Y, Doğer E, Budak O, Cekmen M, Calışkan E. Effect of follicular fluid NO, MDA and GSH levels on in vitro fertilization outcomes. J Turkish Ger Gynecol Assoc 2013;14:136–41. doi:10.5152/jtgga.2013.53323.

[3] Barroso G, Barrionuevo M, Rao P, Graham L, Danforth D, Huey S, et al. Vascular endothelial growth factor, nitric oxide, and leptin follicular fluid levels correlate negatively with embryo quality in IVF patients. Fertil Steril 1999;72:1024–6.

[4] Pacher P, Beckman JS, Liaudet L. Nitric Oxide and Peroxynitrite in Health and Disease. Physiol Rev 2007;87:315–424. doi:10.1152/physrev.00029.2006.

[5] Basini G, Baratta M, Ponderato N, Bussolati S, Tamanini C. Is nitric oxide an autocrine modulator of bovine granulosa cell function? Reprod Fertil Dev 1998;10:471–8.

[6] Romero-Aguirregomezcorta J, Santa ÁP, García-Vázquez FA, Coy P, Matás C. Nitric oxide synthase (NOS) inhibition during porcine in vitro maturation modifies oocyte protein S-nitrosylation and in vitro fertilization. PLoS One 2014;9:e115044. doi:10.1371/journal.pone.0115044.

[7] Goud AP, Goud PT, Diamond MP, Abu-Soud HM. Nitric Oxide Delays Oocyte Aging. Biochemistry 2005;44:11361–8. doi:10.1021/bi050711f.

[8] Anteby EY, Hurwitz A, Korach O, Revel A, Simon A, Finci-Yeheskel Z, et al. Human follicular nitric oxide pathway: relationship to follicular size, oestradiol concentrations and ovarian blood flow. Hum Reprod 1996;11:1947–51.

[9] Barrionuevo MJ, Schwandt RA, Rao PS, Graham LB, Maisel LP, Yeko TR. Nitric oxide (NO) and interleukin-1beta (IL-1beta) in follicular fluid and their correlation with fertilization and embryo cleavage. Am J Reprod Immunol 2000;44:359–64.

[10] Battaglia C, Persico N, Mancini F, De Iaco P, Busacchi P, Facchinetti F, et al. Uterine vascularization and pregnancy outcome in patients undergoing intracytoplasmatic sperm injection: the role of nitric oxide. J Assist Reprod Genet 2006;23:213–22. doi:10.1007/s10815-006-9049-x.

[11] Lee KS, Joo BS, Na YJ, Yoon MS, Choi OH, Kim WW. Relationships between concentrations of tumor necrosis factor-alpha and nitric oxide in follicular fluid and oocyte quality. J Assist Reprod Genet 2000;17:222–8.

[12] Kim KH, Oh DS, Jeong JH, Shin BS, Joo BS, Lee KS. Follicular blood flow is a better predictor of the outcome of in vitro fertilization-embryo transfer than follicular fluid vascular endothelial growth factor and nitric oxide concentrations. Fertil Steril 2004;82:586–92. doi:10.1016/j.fertnstert.2004.02.120.

[13] Manau D, Balasch J, Jiménez W, Fábregues F, Civico S, Casamitjana R, et al. Follicular fluid concentrations of adrenomedullin, vascular endothelial growth factor and nitric oxide in IVF cycles: relationship to ovarian response. Hum Reprod 2000;15:1295–9.

[14] Hakim TS, Sugimori K, Camporesi EM, Anderson G. Half-life of nitric oxide in aqueous solutions with and without haemoglobin. Physiol Meas 1996;17:267–77.

[15] Lundberg JO, Weitzberg E, Gladwin MT. The nitrate–nitrite–nitric oxide pathway in physiology and therapeutics. Nat Rev Drug Discov 2008;7:156–67. doi:10.1038/nrd2466.

[16] Croitoru MD. Nitrite and nitrate can be accurately measured in samples of vegetal and animal origin using an HPLC-UV/VIS technique. J Chromatogr B Analyt Technol Biomed Life Sci 2012;911:154–61. doi:10.1016/j.jchromb.2012.11.006.

[17] Melo M, Busso CE, Bellver J, Alama P, Garrido N, Meseguer M, et al. GnRH agonist versus recombinant HCG in an oocyte donation programme: a randomized, prospective, controlled, assessor-blind study. Reprod Biomed Online 2009;19:486–92.

[18] Ratajczak-Wrona W, Jablonska E, Antonowicz B, Dziemianczyk D, Grabowska SZ. Levels of biological markers of nitric oxide in serum of patients with squamous cell carcinoma of the oral cavity. Int J Oral Sci 2013;5:141–5. doi:10.1038/ijos.2013.59.

[19] Vignini A, Turi A, Giannubilo SR, Pescosolido D, Scognamiglio P, Zanconi S, et al. Follicular fluid nitric oxide (NO) concentrations in stimulated cycles: the relationship to embryo grading. Arch Gynecol Obstet 2008;277:229–32. doi:10.1007/s00404-007-0445-y.

[20] Goud PT, Goud AP, Joshi N, Puscheck E, Diamond MP, Abu-Soud HM. Dynamics of nitric oxide, altered follicular microenvironment, and oocyte quality in women with endometriosis. Fertil Steril 2014;102:151-159.e5. doi:10.1016/j.fertnstert.2014.03.053.

[21] Zhao M, Chang C, Liu Z, Chen LM, Chen Q. The level of vascular endothelial cell growth factor, nitric oxide, and endothelin-1 was correlated with ovarian volume or antral follicle counts: A potential predictor of pregnancy outcome in IVF. Growth Factors 2010;28:299–305. doi:10.3109/08977191003766866.

[22] Revelli A, Delle Piane L, Casano S, Molinari E, Massobrio M, Rinaudo P. Follicular fluid content and oocyte quality: from single biochemical markers to metabolomics. Reprod Biol Endocrinol 2009;7:40. doi:10.1186/1477-7827-7-40.

[23] Shiva S, Wang X, Ringwood LA, Xu X, Yuditskaya S, Annavajjhala V, et al. Ceruloplasmin is a NO oxidase and nitrite synthase that determines endocrine NO homeostasis. Nat Chem Biol 2006;2:486–93. doi:10.1038/nchembio813.

[24] Suchanek E, Mujkic-Klaric A, Grizelj V, Simunic V, Kopjar B. Protein concentration in pre-ovulatory follicular fluid related to ovarian stimulation. Int J Gynaecol Obstet 1990;32:53–9.

[25] Gonzalès J, Lesourd S, Van Dreden P, Richard P, Lefèbvre G, Vauthier Brouzes D. Protein composition of follicular fluid and oocyte cleavage occurrence in in vitro fertilization (IVF). J Assist Reprod Genet 1992;9:211–6.

[26] Gentry PA, Plante L, Schroeder MO, LaMarre J, Young JE, Dodds WG. Human ovarian follicular fluid has functional systems for the generation and modulation of thrombin. Fertil Steril 2000;73:848–54.

[27] Gulamali-Majid F, Ackerman S, Veeck L, Acosta A, Pleban P. Kinetic immunonephelometric determination of protein concentrations in follicular fluid. Clin Chem 1987;33:1185–9.

[28] Cigliano L, Balestrieri M, Spagnuolo MS, Dale B, Abrescia P. Lecithin-cholesterol acyltransferase activity during maturation of human preovulatory follicles with different concentrations of ascorbate, alpha-tocopherol and nitrotyrosine. Reprod Fertil Dev 2002;14:15–21.

[29] Khan FA, Das GK. Follicular fluid nitric oxide and ascorbic acid concentrations in relation to follicle size, functional status and stage of estrous cycle in buffalo. Anim Reprod Sci 2011;125:62–8. doi:10.1016/j.anireprosci.2011.03.012.

[30] Burton GJ, Jauniaux E. Oxidative stress. Best Pract Res Clin Obstet Gynaecol 2011;25:287–99. doi:10.1016/j.bpobgyn.2010.10.016.

[31] Agarwal A, Aponte-Mellado A, Premkumar BJ, Shaman A, Gupta S. The effects of oxidative stress on female reproduction: a review. Reprod Biol Endocrinol 2012;10:49. doi:10.1186/1477-7827-10-49.

[32] Radi R, Beckman JS, Bush KM, Freeman BA. Peroxynitrite-induced membrane lipid peroxidation: the cytotoxic potential of superoxide and nitric oxide. Arch Biochem Biophys 1991;288:481–7.

[33] Panagiotakos DB, Pitsavos C, Arvaniti F, Stefanadis C. Adherence to the Mediterranean food pattern predicts the prevalence of hypertension, hypercholesterolemia, diabetes and obesity, among healthy adults; the accuracy of the MedDietScore. Prev Med (Baltim) 2007;44:335–40. doi:10.1016/j.ypmed.2006.12.009.

CONCLUSIONS

CONCLUSIONS

1. The localization of the nitric oxide synthase isoforms differs according to the sperm regions, the endothelial and neuronal enzymes being mainly located in the sperm head, whereas the inducible isoform in almost all the sperm areas.

2. Nitric oxide regulates the capacitation process in porcine. The inhibition of nitric oxide synthesis in spermatozoa, either through the endothelial and/or inducible isoforms, lowered the straight-line and average path velocity, phosphorylation degree of three Protein Kinase A substrates, acrosome reaction, externalization of phosphatidylserine and intracellular calcium concentration.

3. The penetration rate of cumulus-oocyte complexes and denuded oocytes decreased when nitric oxide synthesis was inhibited during *in vitro* fertilization. This parameter was abolished by the inhibition of the endothelial enzyme in oocytes deprived of cumulus cells.

4. The serine, threonine and tyrosine phosphorylation of four protein bands decreased when the production of nitric oxide was inhibited during human sperm capacitation. The analysis of these bands identified twenty-nine proteins involved in spermatogenesis, binding to the zona pellucida, energy production and metabolism, stress response, motility and structural organization, signaling and protein turnover.

5. The concentrations of nitrite and nitrate can be detected via high performance liquid chromatography in human follicular fluid from oocyte donors and could be used as an indirect measure of the nitric oxide production in this fluid.

6. Although the follicular fluid levels of nitrite and nitrate did not correlate with the yield of total or mature oocytes that were recovered during the stimulation cycles, they were associated with the proportion of mature oocytes.

ABBREVIATIONS

ABBREVIATIONS

$[Ca^{2+}]i$: intracellular calcium concentration;

AC: adenylate cyclase;

AG: Aminoguanidine Hemisulfate salt;

AKAPs: A-Kinase anchoring proteins;

ALH: amplitude of lateral head displacement;

AR: acrosome reaction;

ATP: adenosine triphosphate;

BCF: beat cross-frequency;

BMI: body mass index;

BSA: bovine serum albumin;

Ca^{2+}: calcium;

cAMP: cyclic adenosine monophosphate;

CASA: computer-assisted sperm analysis;

cGMP: cyclic guanosine monophosphate;

CNG: cyclic nucleotide-gated channels;

COCs: cumulus-oocyte complexes;

DPBS: Dulbecco's Phosphate-Buffered Saline without calcium chloride and magnesium chloride;

E2: 17β-estradiol;

eCG: equine chorionic gonadotropin;

eNOS: endothelial nitric oxide synthase;

eNOS-KO: eNOS-knockout;

ERK: extracellular signal regulated kinase;

FF: follicular fluid;

FSH: follicle-stimulating hormone;

GnRH: gonadotropin releasing hormone;

GSNO: S-Nitrosoglutathione;

Hb: hemoglobin;

hCG: human chorionic gonadotropin;

HSPs: heat shock proteins;

IIF: indirect immunofluorescence;

iNOS: inducible nitric oxide synthase;

IVF: *in vitro* fertilization;

IVM: *in vitro* maturation;

kDa: kilodaltons;

LIN: linearity of the curvilinear trajectory;

L-NAME: N^G-Nitro-L-Arginine Methyl Ester Hydrochloride;

nNOS: neuronal nitric oxide synthase;

NO: nitric oxide;

NO_2: nitrite;

NO_3: nitrate;

NOS: nitric oxide synthase;

PBS: phosphate buffer solution without calcium chloride and magnesium chloride;

PDE: cyclic nucleotide phosphodiesterase;

PFF: protein-enriched follicular fluid solution;

PKA: protein kinase A or cAMP-dependent protein kinase;

PKAs-P: phospho-PKA substrates;

PKG: cGMP-dependent protein kinase;

PMCA4: plasma membrane calcium ATPase 4;

PS: phosphatidylserine;

PVA: polyvinyl alcohol;

R: L-Arginine;

RNE: redundant nuclear envelope;

RNS: reactive nitrogen species;

ROS: reactive oxygen species;

RyRs: ryanodine receptors;

SDS: sodium dodecyl sulfate;

SEM: standard error of the mean;

sGC: soluble isoform of guanylate cyclase;

SNP: sodium nitroprusside;

STR: straightness;

TBS: tris-buffered saline;

TPBS: phosphate buffer solution without calcium chloride and magnesium chloride, containing 0.1% *v/v* Tween 20;

TTBS: tris-buffered saline containing 0.1% *v/v* Tween 20;

Tyr-P: tyrosine phosphorylation;

VAP: average path velocity;

VAP: average path velocity;

VCL: curvilinear velocity;

VSL: straight-line velocity;

VSL: straight-line velocity;

WB: Western blot;

WOB: wobble of the curvilinear trajectory;

ZP: zona pellucida;

β-TUB: anti-β-tubulin.