ČESKÁ ZEMĚDĚLSKÁ UNIVERZITA V PRAZE
Fakulta agrobiologie, potravinových a přírodních zdrojů
katedra

Oxidativní stres kančích spermií a možnost jeho modulace gasotransmitery

doktorská disertační práce

Autor: **Ing. Martin Kadlec**

Školitel: **Prof. Ing. Mgr, Markéta Sedmíková, Ph.D.**

Konzultanti: **José Luis Ros-Santaella, Ph.D., Eliana Pintus, Ph.D.**

Praha 2022

Čestné prohlášení

Prohlašuji, že svou disertační práci " Oxidativní stres kančích spermií a možnost jeho modulace gasotransmitery" jsem vypracoval samostatně pod vedením vedoucího práce a s použitím odborné literatury a dalších informačních zdrojů, které jsou citovány v práci a uvedeny v seznamu literatury na konci práce. Jako autorka uvedené disertační práce dále prohlašuji, že jsem v souvislosti s jejím vytvořením neporušila autorská práva třetích osob.

V Praze dne 12.8.2021 _____

Poděkování

Rád bych poděkoval paní profesorce Sedmíkové za podporu a trpělivost během mého studia, díky čemuž jsem mohl rozvíjet své schopnosti nejen v oblasti výzkumu. Také bych rád poděkoval doktorům Josému Luis Ros-Santaella a Elianě Pintus za trpělivost, čas, který mi věnovali, vědomosti, které mi předali, a za jejich inspirativní přístup k vědecké činnosti. Velký dík patří také mým rodičům, bez jejichž podpory bych se neobešel. A konečně musím také poděkovat přítelkyni Haničce Limburské, která mi vytvořila skvělé zázemí pro studium. Společně s mojí dcerou Májou mě jejich neochvějná láska motivovala v průběhu celého studia.

Oxidativní stres kančích spermií a možnost jeho modulace gasotransmitery

Abstrakt

Oxidační stres představuje hlavní překážku v širším využití technik asistované reprodukce v chovu prasat. K oxidačnímu stresu dochází v momentě, kdy přirozené antioxidační systémy nezvládají kontrolovat koncentraci vysoce reaktivních sloučenin. Mezi vysoce reaktivní sloučeniny lze řadit i H_2S a NO, které zároveň spadají do skupiny plynných signálních molekul označované jako gasotransmitery. Účinek NO a H_2S se odvíjí od jejich koncentrace. V rámci fyziologické koncentrace se podílejí na klíčových dějích ve spermii v průběhu jejího životního cyklu. Zvýšená koncentrace těchto gasotransmiterů má naopak fatální dopad na fertilizační potenciál spermie. Pro omezení negativního dopadu oxidačního stresu se využívají antioxidanty, přičemž antioxidační vlastnosti mohou vykazovat i oba gasotransmitery, NO a H_2S, v závislosti na jejich koncentraci.

Pro udržení fyziologické koncentrace NO lze využít aminoguanidin, který inhibuje inducibilní syntázu oxidu dusnatého. Aktivita tohoto enzymu je typická pro leukocyty během zánětlivých reakcí imunitního systému a umožňuje produkci velkého množství NO. K hlavním zdrojům oxidačního stresu v kančím ejakulátu patří právě leukocyty přítomné v semenné plazmě. V této práci aminoguanidin vykazoval silný antioxidační účinek a pomohl ochránit kančí spermie před oxidačním stresem v in vitro podmínkách, což se pozitivně projevilo na funkčních vlastnostech.

Antioxidační vlastnosti byly popsány také u donorů H_2S. V druhé části práce byly porovnány rychlý donor sulfanu, Na_2S, který uvolňuje velké množství H_2S v krátkém časovém úseku, a pomalý donor sulfanu, GYY4317, který uvolňuje menší množství sulfanu v delším časovém úseku. Poprvé byly popsány antioxidační vlastnosti obou donorů, kdy pomalý donor GYY4317 vykazoval silnější antioxidační schopnost, která měla stabilní vzrůstající tendenci. Oproti tomu rychlý donor Na_2S vykazoval menší a nestabilní antioxidační schopnosti. Tomu odpovídali i další výsledky, kdy byl prokázán pozitivní vliv obou donorů sulfanu na funkční parametry spermie. Zatímco GYY4317 vykazoval pozitivní účinek v celém rozsahu koncentrace (3-300 μM), rychlý donor Na_2S vykazoval pozitivní účinek v nízkých mikromolárních koncentracích (3-30 μM) a cytotoxický účinek při 300 μM koncentraci. Tento divergentní účinek rychlého a pomalého donoru H_2S odpovídá povaze vysoce reaktivních sloučenin.

Vzhledem k vysoké reaktivitě obou gasotransmiterů se zdá být pravděpodobná interakce NO s H_2S, která už byla prokázána u somatických buněk. V poslední části práce byla poprvé nepřímo prokázána interakce obou gasotransmiterů za využití SNP (donor NO) a NaHS (rychlý donor H_2S).

Simultánní aplikace 100 SNP nM a 100 nM NaHS vedla k zachování progresivní motility a integrity membrány u kančích spermií v přítomnosti oxidačního stresu. Individuální aplikace těchto donorů NO a H_2S o stejné koncentraci nevykazovala pozitivní účinek na kančí spermie, avšak aplikace SNP vedle k částečnému zlepšení některých funkčních parametrů spermií bez signifikantních výsledků. Lze spekulovat, že interakcí těchto dvou gasotransmiterů dochází k formování metabolitu se silnějším signálním účinkem než samotný NO.

Výsledky disertační práce poskytly originální poznatky v oblasti využití gasotransmiterů při modulaci oxidačního stresu u kančích spermií. Bylo potvrzeno, že jejich účinek je závislý především na koncentraci. Regulací koncentrace gasotransmiterů směrem k fyziologickým hodnotám lze dosáhnout protektivního účinku, kdy si spermie zachovávají funkční vlastnosti a zvyšuje se jejich antioxidační kapacita v in vitro podmínkách. Zároveň je z práce zřejmé, že pro plné pochopení úlohy gasotransmiterů v biologii spermií je zapotřebí dalšího výzkumu.

Klíčová slova: spermie, oxidační stres, gasotransmitery, oxid dusnatý, sulfan

Oxidative stress in boar spermatozoa and its modulation by gasotransmitters

Abstract

Oxidative stress represents a major obstruction in the wide use of assisted reproduction techniques in pig breeding. Oxidative stress is defined as the moment when natural antioxidant systems fail to control the concentration of highly reactive substances. Among such substances can be listed also NO and H_2S which belong to the group of gaseous signalling molecules known as gasotransmitters. The effect of NO and H_2S depends on their concentration. Under physiological concentrations, both gasotransmitters are involved in crucial processes during the life cycle of a sperm cell. On the other hand, elevated concentrations of H_2S and NO result in a detrimental impact on sperm cell fertilization potential. Antioxidants are used to reduce the negative impact of oxidative. Interestingly, both gasotransmitters, NO and H_2S, may exert antioxidant properties depending on their concentration.

Aminoguanidine can be used to maintain the physiological concentration of NO as it inhibits the activity of inducible nitric oxide synthase. The activity of this enzyme is common for leucocytes in the course of an inflammatory reaction of the immunity system and it provides the production of large amounts of NO. The leucocytes present in boar semen constitute one of the major sources of oxidative. This thesis demonstrated the high antioxidant properties of aminoguanidine whose application protected boar spermatozoa from oxidative stress and positively correlated with the functional parameters of sperm cells.

Antioxidant properties were also described for donors of the other gasotransmitter, H_2S. In the second part of the thesis, the fast-releasing H_2S donor (Na_2S) and slow-releasing H_2S donor (GYY4317) were compared. For the first time, the antioxidant properties of both donors were described under in vitro conditions. The slow donor GYY4137 exerted greater and more stable antioxidant capacity with an increasing tendency. On the other hand, the fast donor Na_2S exerted lesser and unstable antioxidant properties. Accordingly, the next results of the thesis proved the positive effect of both donors, NaHS and GYY4137, on the functional parameters of sperm cells. While the fast-releasing donor GYY4137 exerted a positive effect in the whole range of concentration (3-300 μM), the slow-releasing donor Na_2S exerted a positive effect only at low μM concentration and cytotoxic effect at 300 μM concentration. This divergent action of fast- and slow- releasing donors of H_2S corresponds to the nature of highly reactive substances.

Due to the high reactivity of each gasotransmitter, the direct interaction between NO and H_2S seems likely which was proven in somatic cells already. The last section of the thesis has proven

indirectly for the very first time the interaction between the two gasotransmitters using SNP (NO donor) and NaHS (H_2S donor). The sample with these two donors applied simultaneously each at 100 nM concentration led to the preservation of progressive motility and membrane integrity of boar spermatozoa under oxidative stress. Individual application of each donor at the same concentration did not improve sperm quality traits, yet SNP seemed to partially improve some of the functional parameters of boar spermatozoa although the results were not significant. This leads to speculation that metabolites with greater signalling potency are formed upon the interaction of these two gasotransmitters than the sole action of NO alone.

This thesis provided unique findings regarding the application of gasotransmitters for the modulation of oxidative stress in boar spermatozoa. In accordance with the previous knowledge, it was verified that the effect of the two gasotransmitters, NO and H_2S, depends on their concentration. The regulation of these two gasotransmitters towards the physiological levels leads to a protective effect resulting in the preservation of functional properties of boar spermatozoa and the increase of antioxidant capacities under in vitro conditions. The obtained data also underline the need for further investigation regarding the role of gasotransmitters in the biology of sperm cells.

Key words: spermatozoa, oxidative stress, gasotransmitters, nitric oxide, hydrogen sulfide

Obsah

1 Úvod

Moderní chov prasat se převážně opírá o využití asistované reprodukce. Prasnice se obvykle inseminují zchlazenými dávkami určenými pro uchování ejakulátu po dobu maximálně pěti dnů, avšak většina inseminací je prováděna do druhého dne od zhotovení inseminační dávky (Johnson et al., 2006). Dlouhodobější uchovávání kančích inseminačních dávek představuje technologický problém, jelikož kančí spermie jsou zvláště citlivé na chladový šok, který nastává při poklesu teploty pod 15°C. Jako hlavní důvod náchylnosti k degradaci při nízkých teplotách se uvádí specifické složení cytoplazmatické membrány kančí spermie, která je charakterizována vysokým obsahem polynenasycených mastných kyselin (PUFA) (Am-in et al., 2011). V důsledku působení nízkých teplot kančí spermie nevratně ztrácí fertilizační potenciál. Pro zachování fertilizačního potenciálu je potřeba uchovávat kančí ejakulát při mírně snížených teplotách mezi 15-17°C, kdy je buněčný metabolismus pouze částečně omezen a hůře se kontroluje mikrobiální kontaminace v inseminační dávce (Gadea, 2003). Na druhou stranu, výroba inseminačních dávek umožňuje efektivnější využití kvalitních samců, kdy je odebraný ejakulát naředěn pomocí průmyslových ředidel. Tímto způsobem lze inseminovat vícero prasnic genetickým materiálem požadovaného jedince (Gadea, 2003). Avšak výroba inseminační dávky představuje náročný proces pro spermii, která se musí adaptovat na rychle se měnící podmínky. V důsledku naředění ejakulátu spermie nejprve zvyšuje svoji aktivitu a následně ztrácí na pohyblivosti a zároveň dochází k poškození plazmatické membrány. Tento jev se označuje jako „dilution effect“ (Johnson et al., 2006). Zároveň během in vitro skladování při snížených teplotách dochází k produkci a akumulaci reaktivních sloučenin (Leahy et Gadella, 2011). Na trhu je dostupná celá řada komerčních ředidel, které mají za cíl těmto technologickým výzvám čelit skrze specifické složením s obsahem látek, které zvyšují ochranu spermie během skladování. Mezi nejznámějšími lze uvést BTS ředidlo (Beltsville Thawing Solution) charakteristické obsahem malého množství draslíkových iontů, které nahrazují ztrátu intracelulárního draslíku ve spermiích a napomáhají tak k zachování motility během skladování. BTS představuje nejpoužívanějšího zástupce krátkodobých ředidel určených k přechovávání inseminačních dávek po dobu tří dnů. Naopak pro dlouhodobé uchování kančího ejakulátu se využívá ředidlo Androhep, které bylo navrženo pro skladování inseminačních dávek po dobu 4 a více dní. Toto ředidlo je charakteristické obsahem pufru Hepes, který reguluje pH, a vysokým obsahem hovězího sérového albuminu (BSA), který chrání spermie před negativním dopadem reaktivních sloučenin, jež se akumulují v průběhu skladování inseminační dávky a vedou ke vzniku oxidačního stresu (Gadea, 2003). Právě omezení akumulace reaktivních sloučenin a vzniku oxidačního stresu v inseminačních dávkách je předmětem současného výzkumu. Nalezení efektivnějších způsobů ochrany spermií během skladování by umožnilo vývoz inseminačních dávek na delší vzdálenosti a technika inseminace by tak byla dostupnější. Výzkum

zaměřený na látky s antioxidačními vlastnostmi představuje příslib ve zlepšení techniky skladování ejakulátu a zvýšení fertilizačního potenciálu kančích inseminačních dávek. Látky s antioxidačními vlastnostmi lze hledat i mezi signálními plynnými molekulami, gasotransmitery, které se zároveň podílí na klíčových fyziologických procesech ve spermii.

2 Literární rešerše

2.1 Reprodukční charakteristiky kanců

Kanec je znám produkcí velkého množství ejakulátu, jehož objem je obvykle v rozmezí 150 - 300 ml, což je nejvíce ze všech hospodářských zvířat. Z toho vyplývá, že kančí ejakulát je méně koncentrovaný, avšak celkový počet spermií dosahuje až 100 T na jednu dávku. Ejakulace u kanců trvá až třicet minut, což je nesrovnatelně déle než u jakéhokoli jiného hospodářského druhu zvířete (Bonet et al., 2013). Čerstvý ejakulát má za fyziologických podmínek pH v rozmezí 6,85 – 7,9 a lze jej rozdělit na tři frakce: prespermatická, spermatická a postspermatická (Sancho et Vilagran, 2013). První zmíněná frakce neobsahuje spermie a je tvořena sekrety prostaty, Cowperových žláz a seminálních váčků. Objem této frakce je obvykle mezi 10-15 ml. Druhá frakce je nejbohatší na spermie a využívá se k přípravě inseminačních dávek. Její objem činí přibližně 150–200 ml a obsahuje sekrety z prostaty a Cowperových žláz. Postspermiová frakce již obsahuje podstatně méně spermií nežli předchozí frakce, i když je přibližně stejného objemu. Plazma této frakce je tvořena výměšky Cowperových žláz a prostaty a její úkol je podporovat metabolismus spermií v reprodukčním traktu samice. Tudíž není odebírána pro přípravu inseminačních dávek, i když má největší antioxidační kapacitu (Sancho et Villagran, 2013). Nehledě na frakci, hlavní úkolem semenné plazmy je zajištění výživy, ochrany a podpory spermií. Jako hlavní výživovou složku v semenné plazmě kanců lze označit fruktózu, kterou doplňuje glukóza a sorbitol (Peña et al., 2003). Na ochraně spermií se podílí z velké části superoxid dismutáza (SOD), která je na rozdíl od katalázy bohatě zastoupená v kančí semenné plazmě (Kowalowka et al., 2008).

2.1.1 Kančí spermie

Typický vzhled savčí spermie, která se dělí na hlavičku, krček a ocásek, je výsledkem testikulárního vývoje ze zárodečných buněk v semenotvorných kanálcích. Organizace spermie je již zachována až do oplození, avšak fertilizační potenciál získává spermie až v průběhu dalšího vývoje v nadvarleti (Gadella, 2017). Pro testikulární spermii je charakteristická přítomnost cytoplazmatické kapky, jež představuje zbytky organel, kterých se spermie zbavila během spermatogeneze. V případě kančích spermií cytoplazmatická kapka snižuje schopnost pohybu po ejakulaci, a tedy i fertilizační potenciál. Z hlediska oplozovací schopnosti je zásadní přítomnost membránových proteinových komplexů, která podmiňuje schopnost fúze spermie s oocytem (Burkin et Miller, 2000). Testikulární spermie je navíc nepohyblivá a tuto schopnost získává až v průběhu následujícího vývoje (Dacheux et al., 2005). V rámci testikulárního vývoje může rozlišit dvě hlavní fáze: spermatogenezi (spermatocytogenezi) a spermiogenezi (spermiohistogeneze). Formování kančí spermie trvá 34-36

dní, přičemž spermatogeneze zahrnuje postupnou přeměnu samčích zárodečných buněk ve spermatidy. V průběhu spermiogeneze kulaté spermatidy bez bičíku získají svojí definitivní podobu typické savčí spermie (Pinart et al., 1999, 2000).

Samčí zárodečné buňky – spermatogonie, které stojí na počátku spermatogeneze, lze lokalizovat při bazální membráně semenotvorných kanálků ve společnosti Sertoliho buněk zajišťující výživu samčích gamet a organizaci spermatogeneze. Spermatogonie se v následujícím vývoji mění a s každým stádiem se postupně posouvá směrem k apikálnímu konci Sertoliho buňky, kde je na konci vývoje vypuštěna do lumen semenotvorných kanálků a varletní sítí následně odvedena do nadvarlete. Několikanásobné mitotické dělení spermatogonií zajišťuje z jedné části trvalou zásobu kmenových zárodečných buněk (Ljiljak et al., 2012) a z části druhé vede k formování primárních spermatocytů (Garcia-Gil et al., 2002). Primární spermatocyty následně zahajují první meiotické dělení, které vede k formování sekundárních spermatocytů, které jsou již haploidní a podstupují další meiotické dělení (ekvatoriální). Výsledkem jsou čtyři haploidní spermatidy (Garcia-Gil et al., 2002), které následovně zahajují proces spermiogeneze.

Diferenciace kulaté spermatidy ve spermii a její uvolnění do lumen semenotvorného kanálku trvá u kance 14 dní. Diferenciace spermatidy se dělí na čtyři fáze: Golgiho fáze, fáze čepičky, akrozomální fáze a maturační fáze a lze odlišit až 9 vývojových stádií spermatidy v závislosti na zdroji literatury (Bonet et al., 2013). Golgiho fázi charakterizuje přítomnost proakrozomálního lysozomu, který vzniká z Golgiho aparátu, a kondenzace DNA, ve které jsou histony nahrazovány protaminy. Zároveň se začíná tvořit „páteř“ bičíku, axonema. Ve fázi čepičky se proakrozomální lysozom přesouvá nad jádro a vytváří pomyslnou apikální jadernou čepičku. Definitivní diferenciace akrozomu s obsahem typických hydrolytických enzymů (hyaluronidáza, či akrozin) udává jméno třetí, akrozomální, fáze. Během této fáze se reorganizují mikrotubuly v oblasti jádra, kde vzniká perinukleární manžeta, a jádro získává svůj charakteristický protáhlý tvar. Distální centriola se přesouvá k bázi hlavičky, kde se mění v bazální tělísko bičíku. Zároveň dochází k přesunu mitochondrií do proximální části bičíku. Při maturační fázi se formuje mezi hlavičkou a bičíkem proximální cytoplazmatická kapka, která představuje reziduální cytoplazmu (Bonet et al., 2013). Zbytky nepotřebných organel a mezibuněčných spojů jedné kohorty spermií tvoří residuální tělíska, která jsou fagocytována Sertoliho buňkami (O'Donnell et al., 2011).

Po dokončení spermiogeneze jsou nové spermie vypuštěny do lumen semenotvorných kanálků a varletní sítí jsou dopraveny do nadvarlete, kde získávají svůj fertilizační potenciál v procesu zvaném epididymální maturace. Tento proces trvá u kanců přibližně 14 dní (Dacheux et al., 2005). Epididymální maturaci lze charakterizovat rozsáhlými změnami plazmatické membrány. Zároveň se

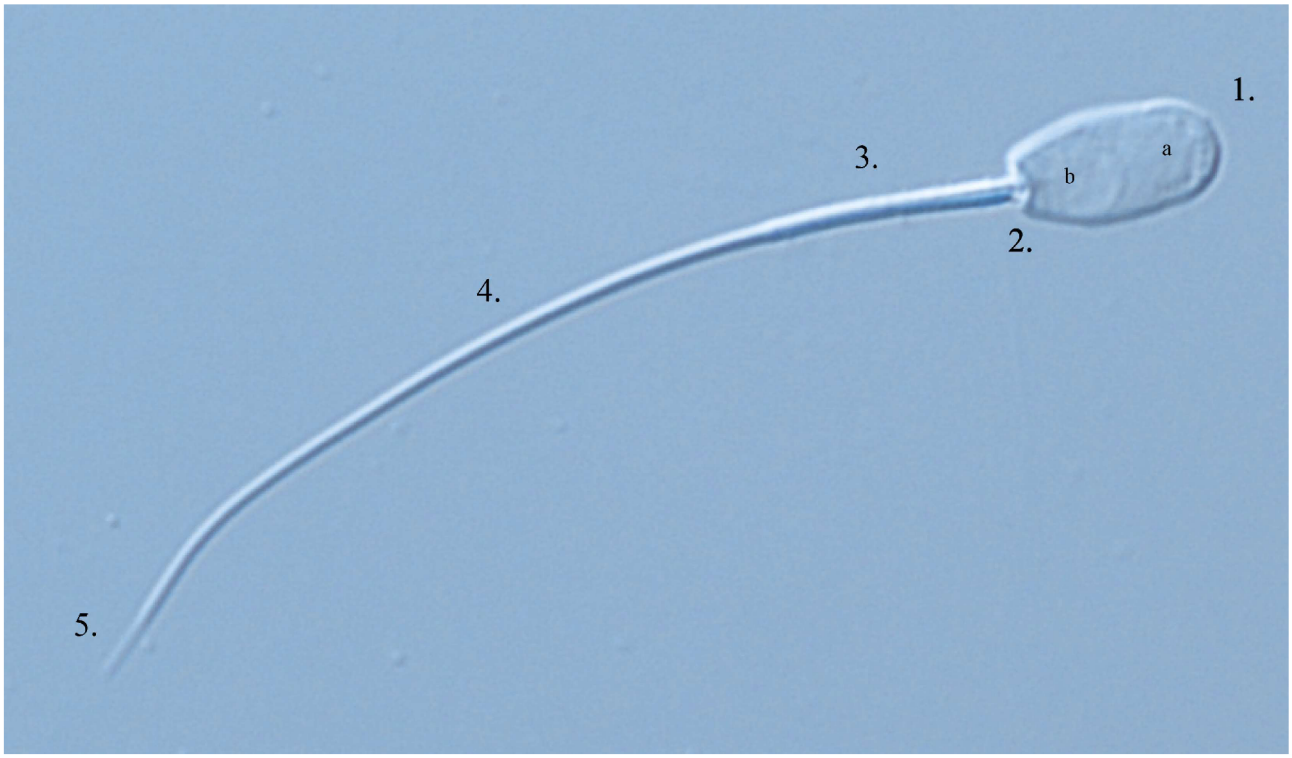
spermie zbavuje cytoplazmatické kapky a mění se její metabolismus, přičemž spermie se stává pohyblivou (Bonet et al., 2013).

Vyzrálá kančí spermie měří přibližně 44 µm (Bonet et al., 2013) a její organizace odpovídá účelu, pro který byla stvořena (Obr. 1). Kromě samčí DNA obsahují spermie pouze několik dalších nezbytných součástí, které umožňují oplození oocytu. Na apikálním konci spermie se nachází pod plazmatickou membránou akrozom, který pokrývá jádro přibližně ze dvou třetin a tvoří tak pomyslnou „čepičku". V akrozomu lze odlišit vnější a vnitřní membránu. Vnitřní membrána pokrývá zhruba apikální část jádra a na vzdálených okrajích přechází ve vnější akrozomální membránu, která leží pod cytoplazmatickou membránou spermie. Mezi vnější a vnitřní akrozomální membránou se nachází akrozomální matrix vyplněná amorfní hmotou s vysokým zastoupením hydrolytických enzymů (Bonet et al., 2013). V post-akrosomálním oddílu se nachází *lamina densa*. Tato homogenní struktura je pevně připojena k cytoplazmatické membráně v místech, které nepokrývá akrozom. Spolu s vnitřní membránou akrozomu tak vymezuje subakrozomální prostor, který obklopuje jádro a je vyplněn fibrózní perinukleární hmotou (Bonet et al., 2013). Samotné jádro měří přibližně 6,6 µm a v případě kančí spermie má ovoidní tvar. Uvnitř jádra se nachází extrémně kondenzovaná chromatinová vlákna.

Na hlavičku spermie navazuje krátký oddíl spojující hlavičku s bičíkem. Krček je dlouhý přibližně 0,7 µm a má lichoběžníkovitý tvar. Širší základna navazuje na hlavičku spermie a je tvořena postcefalickým kroužkem. Krček obsahuje centriolu, která je nezbytná při oplození vajíčka a následné rýhování embrya (Nagy, 2000). Dále lze najít v krčku laminární tělíska, bazální destičky a axonemu, která se táhne dále po celé délce bičíku (Bonet et al., 2013). Bazální destička složením odpovídá *lamina densa* a navazuje na vnější jadernou membránu při kaudálním konci jádra. Laminární tělíska jsou utvořena z přebytečného jaderného obalu a táhnou se od základny jádra a přecházejí v mitochondriální pochvu v proximální části bičíku (Bonet et al., 2013).

Bičík spermie, který má kónický tvar, lze rozdělit na tři hlavní úseky: proximální (spojovací oddíl), hlavní a terminální segment. Po celé délce bičíku se táhne axonema. Tato osa má strukturu charakteristickou pro bičíky a řasinky eukaryotických buněk. V jejím středu se nachází pár mikrotubulů, který je obklopen dalšími devíti páry mikrotubulů (Bonet et al., 2013). Skrze aktivitu dyneinových ramének a nexinových můstku umožňuje pohyb spermie za spotřeby adenosintrifosfátu (ATP) (Mann et Lutwak-Mann, 1981). Ve středovém úseku spermie se pod plazmatickou membránou nachází mitochondriální pochva, která obklopuje mitochondrie uložené spirálovitě kolem axonemy. Mezi mitochondriální pochvou a periferními mikrotubuly axonemy se táhnou hustá vnější vlákna, která zasahují až do hlavní části bičíku. Hlavní část bičíku začíná Jensenovým prstencem (*anulus*), který navazuje na poslední mitochondrie ve středovém oddílu bičíku. Mitochondriální pochva zde

přechází ve fibrózní pochvu. Vnější hustá vlákna se postupně ztenčují a končí přibližně v první třetině hlavního segmentu. Terminální oddíl je tvořen pouze axonemou, kterou přímo pokrývá plazmatická membrána spermie (Bonet et al., 2013).



Obrázek 1. Kančí spermie. **1:** Hlavička; **a:** akrozomální segment; **b:** post-akrozomální segment. **2:** krček; **3:** proximální oddíl bičíku; **4:** hlavní část bičíku; **5:** terminální část bičíku (Kadlec, 2022).

Plazmatická membrána spermie

Jestliže je zralá samčí pohlavní buňka typická malým počtem organel v porovnání se somatickou buňkou, pak je výrazně komplexnější, co se týče složení plazmatické membrány. Vzhledem k různorodému zastoupení lipidů a proteinů lze charakterizovat plazmatickou membránu spermií vysokým stupněm molekulárního mozaicismu (Bonet et al., 2013). Navíc plazmatická membrána zralé spermie prochází rozsáhlými změnami v reprodukčním traktu samice (Brewis et Gadella, 2009), kde se dále mění zastoupení proteinů a lipidů (Flesch et Gadella, 2000).

Specifické složení plazmatické membrány kančí spermie do značné míry ovlivňuje její možnosti využití technik asistované reprodukce u prasat. Plazmatickou membránu kančí spermie lze rozdělit do pěti hlavních domén (Bonet et al., 2013), z nichž tři jsou přítomné na hlavičce: apikální, pre-ekvatoriální a ekvatoriální doména hlavičky. Jak bylo uvedeno výše, apikální doména slouží

k rozpoznání a navázaní spermie na vajíčko, pre-ekvatoriální umožňuje akrozomální reakci a ekvatoriální doména umožňuje fúzi spermie s vajíčkem (Brewis et Gadella, 2009). Na bičíku spermie lze odlišit další dvě domény, jedna pokrývá středový segment bičíku, druhá pokrývá hlavní část bičíku. Význam těchto dvou domén nebyl objasněn, předpokládá se, že napomáhají ke správnému pohybu spermie (Brewis et Gadella, 2009). Rozdělení plazmatické membrány spermie do domén umožňuje nezávislou interakci struktur, které pokrývá (Brewis et Gadella, 2009). Plazmatická membrána kančí spermie obsahuje vysoký podíl fosfolipidů s navázaným esterem a lipidů obsahujících polynenasycené alifatické řetězce (Evans et al., 1980). Na druhou stranu membrána kančích spermií obsahuje relativně málo cholesterolu. Fosfolipidy představují 70 % z celkových lipidů membrány, přičemž nejvíce zastoupen je cholin a dále také steroidy (Nikolopoulou et al., 1985). Zbylé membránové lipidů zastupují především neutrální tuky, glykolipidy jsou nejméně zastoupené (okolo 5 %) (Mann et Lutwak-Mann, 1981). Právě kvůli vysokému obsahu polynenasycených mastný kyselin (PUFA) ve fosfolipidech membrány je kančí spermie náchylná na lipidovou peroxidaci způsobenou reaktivními formami kyslíku (ROS) (Am-in et al., 2011). Lipidová peroxidace plazmatické membrány pak dále zvyšuje produkci ROS a tím i umocňuje negativní dopad oxidačního stresu na fertilizační potenciál spermie (Aitken, 1995).

2.2 Oxidační stres

Jako oxidační stres označujeme stav, kdy se zvedne koncentrace reaktivních sloučenin nad fyziologickou míru. Akumulované reaktivní sloučeniny již nadále neplní fyziologickou funkci a místo toho poškozují veškeré buněčné komponenty od lipidů a proteinů plazmatické membrány, přes cytoskeletální struktury a axonemu až po samotnou DNA v jádru spermie (Cerolini et al., 2000). K nadměrné produkci reaktivních sloučenin přispívají zdroje endogenní a exogenní (Toor et Sikka, 2019). Jako hlavní endogenní zdroj reaktivních sloučenin se uvádí spermie, které disponují několika mechanismy vedoucími k produkci reaktivních sloučenin. Oxidativní fosforylace probíhající na vnitřní membráně mitochondrií ve středovém oddílu bičíku vede k produkci superoxidového anionu (O_2^-), který lze označit za primární ROS. V případě defektních spermií s poškozeným středovým segmentem bičíku spermie spontánně produkují kyslíkaté radikály mitochondriálního původu (Aitken et al., 2016). K nadměrné akumulaci reaktivních sloučenin také přispívají nezralé spermie s nadbytečným množství residuální cytoplasmy (Aitken, 1995). Reziduální cytoplasma okolo středového segmentu bičíku obsahuje zvýšenou koncentraci glukózo-6-fosfát dehydrogenázy (G6PDH), která vede ke vzniku nikotinamidadenindinukleotid fosfátu (NADPH). Při následné reakci s NAPDH oxygenázou vzniká značné množství O_2^- (Agarwal et al., 2018). Ke zvýšené koncentraci ROS v ejakulátu přispívá i přítomnost mrtvých spermií skrze aktivitu oxidáz aromatických

aminokyselin (Bansal et Bilaspuri, 2011). Jako nejvýznamnější exogenní zdroj reaktivních sloučenin lze uvést leukocyty přítomné v semenné plazmě (Toor et Sikka, 2019), které mají původ v přídatných pohlavních žlázách (Otasevic et al., 2020). Z leukocytů jsou zastoupeny především makrofágy a neutrofily (Bansal et Bilaspuri, 2011). Jako významný exogenní zdroj oxidačního stresu se považuje také manipulace s ejakulátem v *in vitro* podmínkách, kdy dochází ke zvýšené produkci reaktivních sloučenin v důsledku teplotních změn, změnám metabolismu spermií v důsledku ředění, či vystavení UV záření (Esfandiari et al., 2002; Toor et Sikka, 2019).

2.3 Reaktivní sloučeniny a oxidační stres

Mezi reaktivní sloučeniny, které se podílejí na vzniku oxidačního stresu se řadí i reaktivní formy dusíku (RNS), u kterých lze jako výchozí radikál označit oxid dusnatý. Reakcí oxidu dusnatého s ROS vzniká peroxynitrit ($ONOO^-$), který je více reaktivní a jeho akumulace způsobuje peroxidaci lipidů a vede k poškození plazmatické membrány. Zároveň již v μM koncentracích inhibuje mitochondriální aktivitu skrze inhibici I. (NADP dehydrogenáza) a II. (sukcionát dehydrogenáza) mitochondriálního komplexu. Také inhibuje aktivitu SOD skrze nitrataci tyrosinu, což vede nejen ke snížení motility spermií, ale může také způsobovat buněčnou apoptózu (Otasevic et al., 2020). Další významné zástupce vysoce reaktivních látek lze najít mezi reaktivními sloučeninami síry (RSS), přičemž jako výchozí molekulu lze označit sulfan (H_2S). Aktuální definice RSS zahrnuje širokou škálu reaktivních sloučenin obsahujících síru, pro které je společná definice: „Reaktivní formy síry lze popsat jako molekuly, které obsahují alespoň jeden redoxně aktivní atom síry, nebo funkční skupinu obsahující síru ve své struktuře a zároveň jsou schopné buďto oxidovat či redukovat biologicky aktivní molekuly v rámci fyziologických podmínek, což vede ke spuštění či propagaci buněčné signalizace či jiné významné události v buňce (Giles et al., 2017). Mishanina et al. (2015) uvádí rozsáhlý seznam biologicky aktivních RSS, které mají společný prekurzor, H_2S. Stejně jako v případě ROS a RNS, nadměrná akumulace RSS má fatální účinek na kvalitu spermie. Zhao et al. (2016) prokázali negativní dopad zvýšené koncentrace H_2S na motilitu spermií. Tento efekt lze přisuzovat snížené produkci ATP v důsledku blokování IV. mitochondriálního komplexu při suprafyziologické intracelulární koncentraci H_2S. Stejně jako ROS a RNS, akumulace reaktivních sloučenin síry vede ke zvýšení oxidačního stresu, což se projevuje i zvýšením lipidové peroxidace plazmatické membrány.

Rozdíl mezi oxidačním stresem a fyziologickou aktivitou RS se odvíjí pouze od jejich koncentrace. Vzhledem k minimálnímu množství cytoplazmy je však buněčná ochrana spermie značně limitovaná a hranice mezi patologickou a fyziologickou hranicí je velmi tenká (Cristian O'Flaherty, 2014). V případě RNS a RSS se fyziologická koncentrace pohybuje

v rozmezí nanomolárních hodnot (Cooper et Brown, 2008), kdy se reaktivní sloučeniny podílejí na klíčových procesech, kterými musí spermie projít, aby získala plný fertilizační potenciál (Aitken et al., 2012).

2.4 Reaktivní sloučeniny a fyziologické procesy ve spermii

V rámci fyziologických koncentrací se RS podílejí na důležitých procesech ve spermii, jako je kapacitace, hyperaktivace či akrozomální reakce (Obr. 2) (Otasevic et al., 2020).

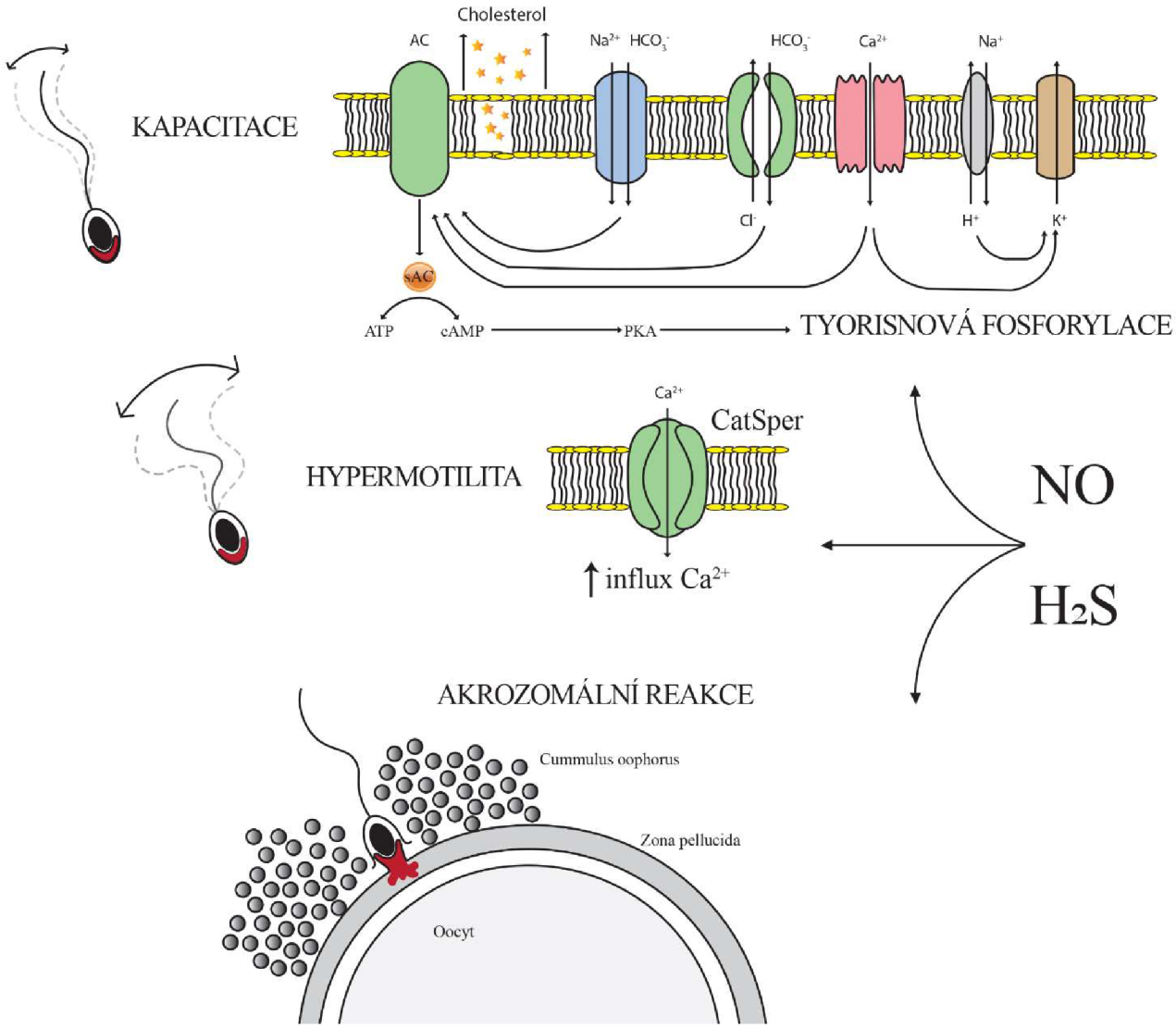
Kapacitace si lze představit jako finální fázi maturace spermie, kterou buňka prodělává v samičím reprodukčním traktu. Kančí spermie se před ovulací hromadí v děložním istmu, kde se tvoří jejich rezervoár a spermie zde postupně kapacitují v průběhu několika hodin. Po kapacitaci jsou spermie uvolněny z istmu, vždy přibližně 5 % rezervoáru, a směřují k ampuli vejcovodu, kde dochází k oplození. Tak je zajištěn trvalý přísun spermií připravených k oplození a zvyšuje se šance na fertilizaci oocytu (Bonet et al., 2013). Během samotné kapacitace je z membrány spermie uvolněn cholesterol, zvyšuje se její fluidita, mění se koncentrace intracelulárních iontů (Ca^{2+}) v důsledku čehož nastává hyperpolarizace membrány, aktivizuje se protein kináza A (PKA) a proteiny spermie jsou fosforylovány tyrosinem (Stival et al., 2016). V případě kančí spermie kapacitaci navozuje bikarbonát (HCO_3^-) (Vicente-Carrillo et al., 2017), jehož zvýšená koncentrace v seminální plazmě aktivuje spermie, které byly v klidovém stavu skladovány v epididymu. V době ovulace se koncentrace HCO_3^- uvnitř samičího traktu dále zvyšuje a iniciuje kapacitaci (Stival et al., 2016), která u prasete trvá přibližně 2 hodiny (Bonet et al., 2013). ROS během kapacitace inhibují aktivitu tyrozinové fosfatázy a stimulují aktivitu adenylát cyklázy a tím napomáhají aktivaci PKA. ROS se také podílejí na oxidaci cholesterolu, která vede k jeho efluxu z plazmatické membrány (Aitken, 2017; Herrero et al., 2000; de Lamirande et O'Flaherty, 2008; Lopez, 2015).

V důsledku kapacitace se mění metabolismus spermie a ta se stává hyperaktivní. Její pohyb je intenzivnější, což se projevuje větším vychýlením hlavičky a širší amplitudou pohybu ocásku. Hyperaktivní spermie má větší šanci dosáhnout oocytu a proniknout granulárními buňkami obklopujícími vajíčko. Na hyperaktivaci se přímo podílí reaktivní sloučeniny, které skrze tyrosinovou fosforylaci modulují pohyb bičíku (Suarez et Ho, 2003).

Po navázání spermie na zonu pellucidu oocytu, spermie začínají uvolňovat proteolytické enzymy z akrozomu, tento děj se označuje jako akrozomální reakce. Proteolytické enzymy narušují zonu pellucidu a umožňují průnik a fúzi spermie s vajíčkem (Otasevic et al., 2020). Akrozomální reakce je spuštěna ZP3 proteinem, který se nachází na zoně pellucidě. Jedná se o redoxně regulovaný proces, na kterém se také podílejí ROS, jejichž přidání do kultivačního média spouští akrozomální reakci (de Lamirande et O'Flaherty, 2008). ROS aktivují klíčové enzymy, které se podílejí na

akrozomální reakci (e.g. solubilní guanylát cykláza; sGC) (Aitken, 2017; Aquila et al., 2011; Lopez, 2015). Na všech klíčových procesech ve spermie se podílí zástupci všech RS, přičemž nejvíce prostudované jsou ROS.

V případě ROS lze považovat za hlavní zdroje NADPH oxidázu (NOX), která v přítomnosti kyslíku oxiduje nikotinamid adenin dinukleotid fosfát za vzniku superoxidu (O_2^-). Další významný zdroj představují mitochondrie, které produkují superoxid jakožto vedlejší metabolit dýchacího řetězce (Aitken, 2017). Jako alternativní zdroje ROS lze uvést lipoxygenázu, či oxidázu L-amino kyseliny. Zvýšená produkce ROS vede k aktivaci solubilní formy adenylyl cyklázy (sAC), která zajišťuje produkci cyklického adenosinmonofosfátu (cAMP). V důsledku zvýšené koncentrace cAMP se aktivují PKA a tyrosinové kinázy a tím dochází k tyrosinové fosforylaci klíčových proteinu ve spermii. Superoxid se také podílí na oxidaci cholesterolu za vzniku oxysterolů, které opouštějí membránu a tím zvyšují její fluiditu (Aitken, 2017). Část superoxidu je redukována SOD za vzniku H_2O_2, který blokuje aktivitu tyrosinových fosfatáz a umožňuje tak dramatický nárůst tyrosinové fosforylace. Ve stejném smyslu účinkuje také PKA, která aktivuje SRC kinázy, které jsou odpovědné za fosforylaci (inaktivaci) tyrosinových fosfatáz a tím napomáhají tyrosinové fosforylaci. V současné době je již prokázáno, že superoxidový anion interaguje oxidem dusnatým a sulfanem. Společně se tak ROS, RNS a RSS podílejí na regulační procesech spojenými s kapacitací, hyperaktivací a akrozomální reakcí (Aitken, 2017; Otasevic et al., 2020). Na rozdíl od ROS, výchozí sloučeniny RNS a RSS, oxid dusnatý a hydrogen sulfid, vykazují specifické vlastnosti, na základě, kterých vzniklo označení gasotransmitery, které sdružuje plynné signální molekuly.



Obrázek 2. Klíčové děje ve spermii: kapacitace, hyperaktivace a akrozomální reakce. Kapacitace začíná vyvázáním cholesterolu z plazmatické membrány spermií. Následná změna fluidity membrány a aktivace klíčových iontových kanálů pomocí HCO_3^-, jehož koncentrace se v reprodukčním traktu samice dále zvyšuje, vede ke změně membránového potenciálu, aktivaci napětím řízených kanálů, influxu Ca^{2+} a následné aktivaci transmembránové adenylát cyklázy respektive sAC. Ta zajišťuje tvorbu cAMP, který spouští tyrosinovou fosforylaci klíčových proteinů a následně se aktivizuje metabolismus spermie. Počáteční influx Ca^{2+} iontů je dále navýšen aktivací CatSper iontových kanálů, díky kterým je zajištěn dostatečný přísun Ca^{2+} iontů pro hyperaktivaci spermie. Její pohyb se stává intenzivnější a zvyšuje se šance na proniknutí kumulárními buňkami. Po navázání na zonu pellucidu vajíčka dochází k akrozomální reakci. Enzymy v akrozomu umožňují průnik skrz zonu a následnou fúzi gamet. Na všech těchto dějích se podílí i gasotransmitery NO a H_2S (Kadlec, 2022).

2.5 Gasotransmitery

2.5.1 Oxid dusnatý

Za produkci reaktivních forem dusíku je odpovědná syntáza oxidu dusnatého (NOS) u které byly identifikovány tři základní isoformy: neuronální (nNOS), endoteliální (eNOS) a inducibilní (iNOS); jako hlavní substrát pro aktivitu NOS slouží L-arginin a kyslík (Förstermann et Kleinert, 1995). Aktivitou NOS vzniká oxid dusnatý, který představuje výchozí reaktivní sloučeninu dusíku (Türkyilmaz et al., 2004). První dvě zmíněné isoformy, nNOS a eNOS, se označují jako konstitutivní NOS a jsou aktivovány Ca^{2+} a kalmodulinem, přičemž je charakterizuje kontinuální uvolňování malých koncentrací NO (Herrero et Gagnon, 2001). Neurální isoforma byla poprvé identifikována v neuronech centrální nervové soustavy, kde se podílí na regulaci synaptické aktivity. Následně bylo zjištěno, že ovlivňuje krevní tlak skrze relaxaci hladké svaloviny stejně jako eNOS, která byla poprvé identifikována v endoteliálních buňkách. Naopak iNOS byla identifikována poprvé u makrofágů, které produkují jednorázové cytotoxické množství NO, a tak kontrolují buněčný růst a podílejí se na odstraňování cizorodých mikroorganismů v těle. Aktivita iNOS je indukována lipopolysacharidy a cytokiny (Lind et al., 2017). Aktivitou iNOS v makrofázích tedy vznikají „vzplanutí“ oxidu dusnatého při zánětlivých reakcích, a proto lze iNOS považovat za důležitou součást imunitního systému (García-Ortiz et Serrador, 2018; Lundberg et al., 2015). V pozdějším výzkumu byla prokázána přítomnost iNOS ve většině tkání včetně reprodukčního systému, kde se podílí na fyziologických procesech (Herrero et Gagnon, 2001). Nedávná studie (Staicu et al., 2019) prokázala přítomnost všech tří isoforem NOS v kančích spermiích. Zatímco eNOS a nNOS byly přítomné v hlavičce spermie, iNOS byla lokalizována v bičíku. Studie také naznačuje souvislost mezi distribucí isoforem NOS a normální funkcí spermie. Zároveň se zdá, že distribuce NOS je druhově specifická, kdy u kočičích spermií jsou všechny tři isoformy přítomné v bičíku a cytoplazmatické kapce (Liman et Alan, 2016). Oxid dusnatý produkovaný isoformami NOS aktivuje sGC, jejíž aktivita zvyšuje koncentraci cyklického guanylát monofosfátu (cGMP). Navázáním cGMP na CNG (cyclic nucleotid gated) iontové kanály v membráně bičíku je regulován influx Ca^{2+} iontů v průběhu kapacitace. Zvýšená koncentrace cGMP také vede k aktivaci cGMP-dependentní protein kinázy (PKG), která fosforyluje serin a treonin proteinů klíčových pro kapacitaci a zároveň PKG aktivuje makroskopické iontové kanály odpovědné za dlouhodobé udržování vysoké intracelulární hladiny Ca^{2+} iontů během kapacitace (Cisneros-Mejorado et al., 2014). Zvýšená hladina cGMP také nepřímo zvyšuje hladinu cAMP ve spermii, jelikož se také váže na fosfodiesterázu typu 3 (PDE3), která primárně degraduje cAMP (Staicu et al., 2019). Při vysokých hladinách NO dochází také k tyrosinové nitraci a S-

nytrosilaci, přičemž bylo identifikováno na 200 proteinů v lidské spermii, které představují potenciální cíle signalizace NO (Lefièvre et al., 2007).

2.5.2 Sulfan

Existuje celá řada biologicky aktivních reaktivních sloučenin obsahujících síru, pro které je společný jeden prekurzor, sulfan (Mishanina et al., 2015). Cílená produkce sulfanu v buňce je primárně zajištěna třemi enzymy: cystationin β-syntazou (CBS), cystationin γ-lyazou (CSE) a 3-merkaptopyruvat sulfutransferazou (3-MST). Jak CBS, tak CSE se nacházejí převážně v cytosolu a funkce těchto enzymů je dependentní na dostupnosti pyridoxal 5-fosfátu. Naopak aktivita 3-MST je podmíněna přítomností zinku a nachází se především v mitochondriích (Kolluru et al., 2017). Za určitých okolností může být CSE přesunuta z cytosolu do mitochondrií, kde vytváří H_2S a zároveň napomáhá ke zvýšení produkce ATP (Fu et al., 2012). Společným substrátem pro sulfan-produkující enzymy jsou L-homocystein a L-cystein, které vznikají metabolicky transulfurací metioninu, nebo jsou přijímány přímo v potravě (Olson et Straub, 2016). Alternativní zdroj H_2S představuje metabolismus α-ketoglutarátu (Olson, 2018). Produkce H_2S aktivitou 3-MST je primárně spojena s metabolickou cestou zahrnující cystein aminotransferázu (CAT) a α-ketoglutarát, avšak alternativní metabolická cesta zahrnuje oxidázu D-aminokyselin (DAO) a D-cystein (Olson, 2019). Enzymatická produkce sulfanu může být také zajištěna redukcí tiolů katalázou (Olson et al., 2017), která navíc může oxidovat H_2S. Proto lze předpokládat, že kataláza hraje významnou roli v buněčném metabolismu H_2S (Olson, 2019). Významný alternativní zdroj H_2S představuje I. mitochondriální komplex, který je charakteristický relativně vysokou koncentrací cysteinu v porovnání s cytosolem. Buněčný katabolismus sulfanu zatím není prostudován (Kimura, 2014), avšak zdá se být spojený především s mitochondriemi, kde se nacházejí enzymy schopné oxidace H_2S: sulfid chinon oxidoreduktáza (SQR), thiosulfát transferáza (TST) a sulfid oxidáza (Olson, 2018). Na aktivitu SQR navazuje ethylmalon-encefalopatický protein 1 (ETHE1), který dále oxiduje sulfidy (Rose et al., 2017). Zdá se, že na katabolismu H_2S se podílejí i ROS a RNS (O_2^-, H_2O_2, $ONOO^-$), které H_2S oxidují (Olson, 2018).

2.6 Interakce NO a H_2S

Jsou to právě interakce mezi gasotransmitery a jejich produkty, o které se aktuálně zajímá výzkum zaměřený na jejich úlohu v somatických buňkách těla. Rostoucí množství studií věnujících se kardiovaskulární soustavě přináší důkazy o interakcích NO a H_2S, které probíhají na několika úrovních. Mezi NO a H_2S existují společné cíle buněčné signalizace, zároveň můžou oba

gasotransmitery vzájemně ovlivňovat svůj metabolismus a také se předpokládá interakce metabolitů obou gasotransmiterů (Nagpure et Bian, 2016).

Ze společných buněčných cílů signalizace lze uvést například mitogenem aktivované protein kinázy (MAPK), mezi které řadíme kaskádu extracelulárně regulované kinázy (ERK) 1/2, C-Jun N-terminální kinázu (JNK), p38 a ERK 5. Recentní studie dokazují, že oba gasotransmitery ovlivňují funkci těchto kináz, které se účastní kapacitace, akrozomové reakce a ovlivňují motilitu spermií (Almog et Naor, 2008). Zatímco H_2S snižuje fosforylaci MAPK ve varlatech (Wang et al., 2018), NO naopak aktivuje MAPK v Sertoliho buňkách (Lee et Cheng, 2004). Antagonistická regulace MAPK v buňkách reprodukční soustavy samců je zajímavá ve světle studií, které dokazují vliv MAPK na motilitu, morfologii a kapacitaci spermií (Gangwar et Atreja, 2015; Silva et al., 2015). Silva et al. (2015) poprvé u lidských spermií identifikovali JNK, které spadají do podskupiny MAP kináz označovaných jako stresem aktivované protein kinázy (SAPKs), k jejichž aktivaci fosforylací dochází při oxidačním stresu. V této studii byla zdokumentována negativní korelace hladiny fosforylované JNK a progresivní motility. Jiná studie od Wang et al. (2018) prokázala sníženou aktivitu MAPK v testikulární krevní bariéře myších varlat vystavených oxidačnímu stresu, který byl indukován pomocí lipopolysacharidů (LPS). Na základě těchto výsledku lze usuzovat, že H_2S snižuje fosforylaci MAPK. Naopak zvýšená fosforylace aktivující MAPKs byla pozorována po vystavení epiteliálních buněk potkaních jater peroxynitritu (Schieke et al., 1999). Nicméně efekt působení obou gasotransmiterů na MAPK ve spermii stále nebyl ověřen. Další společný cíl buněčné signalizace představují Ca^{2+} iontové kanály (Kolluru et al., 2017; Shefa et al., 2018) a K^+ iontové kanály (Kolluru et al., 2017; Nagpure et Bian, 2016; Shefa et al., 2018). Právě regulace Ca^{2+} iontových kanálů je významná pro funkci spermie. Například CatSper iontové kanály se podílí nejen na kapacitaci (Molina et al., 2018), ale také na hyperaktivaci, akrozomální reakci a chemotaxi (Miki et Clapham, 2013; Singh et Rajender, 2015). Miraglia et al. (2007) ve své studii uvádí hypotézu, že positivní efekt NO na migraci spermií byl výsledkem aktivace iontových kanálů. Na druhou stranu Wiliński et al. (2015) zaznamenal dočasné zpomalení chemotaxe u spermií vystavených H_2S, pravděpodobně v důsledku vysoké koncentrace H_2S a snížené motility. Mezi Ca^{2+} iontové kanály se řadí také TRP (transient receptor potential) iontové kanály, kam spadá 30 rozdílných Ca^{2+} kanálů tvořících 7 skupin a jež se účastní termotaxe spermií (Björkgren et Lishko, 2017; Kumar et al., 2018). Podskupina TRP iontových kanálů, TRP vanilloid (TRPV) iontové kanály, je aktivována H_2S a NO (Kolluru et al., 2017; Yoshida et al., 2006). Nedávno bylo zjištěno, že TRPV typ 4 se podílí na kapacitaci a hyperaktivaci lidských spermií (Mundt et al., 2018). Ovlivňují influx Na^+, který vede k depolarizaci membrány a následné aktivaci napětím řízeným kanálů (Mundt et al., 2018), přičemž imunolokalizace odhalila přítomnost těchto kanálů na bičíku a akrozomu lidských spermií (Mundt et al., 2018). U

býčích spermií byla prokázána přítomnost TRPV 1 kanálu, přičemž výsledky ukazují na souvislost aktivity TRPV 1 s motilitou, hyperaktivací, kapacitací a akrozomální reakcí. Zároveň TRPV 1 kanál hraje významnou roli při kapacitaci kančích spermií (Bernabò et al., 2010). Bernabò et al. (2010) zaznamenali změnu v rozložení TRPV 1 kanálů po kapacitaci, kdy se TRPV 1 kanály přesunuly z postakrozomální oblasti směrem k akrozomu a středovému oddílu bičíku, kde se nacházejí mitochondrie. Na kapacitaci se podílejí také K^+ iontové kanály, které navozují hyperpolarizaci membrány. K^+ iontové kanály mají také vliv na funkci mitochondrií, a tím pádem ovlivňují i produkci ATP v buňce. Aktivací K^+ iontových kanálů se zvyšuje produkce ATP, což vede ke zvýšení motility a hyperaktivaci (Gupta et al., 2018).

Z hlediska interakcí mezi oběma gasotransmitery, NO a H_2S, je zajímavá schopnost H_2S aktivovat fosfatidylinositol-3-kinázu (PI3K) a ERK v kančích spermiích (Zhao et al., 2016). Právě v případě enzymů ERK byla pozorována schopnost zvýšit citlivost eNOS na stimulaci Ca^{2+} v endoteliálních buňkách (Di et al., 2001). Obdobně, za využití vícero donorů H_2S u myší s vyřazeným genem pro CSE byla aktivována eNOS v myokardiálních buňkách (King, 2013). Ke zvýšení produkce NO po fosforylaci serinu 1179 eNOS na základě aktivity MEK/ERK1/2 a PI3K/akt dochází také po aplikaci H_2O_2 (Cai et al., 2003) a pravděpodobně se jedná o adaptační mechanismus buňky na oxidační stres. Opačně k efektu pozorovaném u eNOS, po aplikaci H_2S donorů (NaHS a diallyl trisulfidu) se inhibuje iNOS během zánětlivé reakce (Benetti et al., 2013). Nicméně stejně jako v předchozím případě, výsledný efekt interakce NO a H_2S je stále nejasný nehledě na typ buňky (Kolluru et al., 2015).

Významná se zdá být i přímá interakce mezi NO a H_2S a jejich metabolity, která vede k formování potenciálně významných signálních molekul jako je například nitroxyl (HNO) (Ivanovic-Burmazovic et Filipovic, 2019; Lefièvre et al., 2007). Současný výzkum se zaměřuje právě na toto komplexní téma se značným významem pro buněčnou fyziologii (Cortese-Krott et al., 2015). Ku příkladu HS^- reaguje s peroxynitritem za vzniku HSNO (Nagpure et Bian, 2016), který představuje potenciálně významný zdroj jak NO, tak HNO (Filipovic et al., 2012). Výzkum zaměřený na efekt HNO v buněčné fyziologii kardiovaskulárního systému odhalil zajímavé výsledky potenciálně relevantní také pro fyziologii spermií (Nagpure et Bian, 2016). Za využití donoru HNO, Andrews et al. (2015) prokázali, že HNO účinkuje skrze aktivaci sGC/cGMP. HNO také vykazuje antioxidační vlastnosti, kdy brání lipidové peroxidaci PUFA (Bianco et al., 2017). Schopnost redukovat lipidovou peroxidaci membrány je potenciálně významná také z hlediska kančích spermií, jejichž membrána je bohatá na PUFA (Bonet et al., 2013). Dále bylo prokázáno, že HNO může bránit degradaci H_2O_2, a tak zvyšovat jeho hladiny, a zároveň reaguje s proteiny obsahujícími thiol, jako je například glyceraldehyd 3-fosfát dehydrogenáza (GAPDH), což vede ke snížení aktivity těchto

enzymů (Andrews et al., 2015; Bianco et al., 2017). Ve spermiích byla identifikována specifická forma GAPDH (GAPDS), která hraje významnou úlohu v energetickém metabolismu spermie (Miki et al., 2004). Jedna z navrhovaných interakcí mezi NO a H_2S vede k formovaní S-nitrosoglutathionu (GSNO) (Broniowska et al., 2013). který se zdá být intracelulárním rezervoárem NO. Reakcí GSNO s GPx, či thioredoxinovou reduktázou se uvolňuje NO. Stejný výsledek se předpokládá i u reakce GSNO a H_2S či HS^- (Ondrias et al., 2008). Dále se také předpokládá, že reakcí GSNO se sulfidy dochází k formování HNO (Berenyiova et al., 2015). Ve vaskulárním systému byla pozorována inhibice NADPH oxidázy (Nox 2) (Nagpure et Bian, 2016), avšak typ a úloha NADPH oxidázy u spermií zůstává neobjasněna (Toor et Sikka, 2019). Prozatím byla identifikována pouze isoforma Nox 5 v lidských varlatech a spermiích, ve kterých byla lokalizována na akrozomu a bičíku a její exprese měla pozitivní vliv na motilitu (Musset et al., 2012). Nedávno byl navržen nitrosopersulfid ($SSNO^-$) jako odolnější a efektivnější donor NO, než GSNO (Cortese-Krott et al., 2015), přičemž k formování $SSNO^-$ vede nadbytečné množství sulfidu (Cortese-Krott et al., 2014). Krom zmíněných interakcí metabolitů NO a H_2S byly navrženy další varianty signalizace těchto dvou gasotransmiterů (Wedmann et al., 2017), což dokazuje komplexnost toho tématu a zároveň podtrhává potřebu dalšího výzkumu účinku simultánních aplikace těchto dvou gasotransmiterů. Hlavní komplikací při určování osudu těchto dvou messengerů v buňce zůstává fakt, že většina metabolitů má krátký poločas rozpadu a neexistují dostatečně specifické sondy pro jejich detekci (Smulik-Izydorczyk et al., 2018). Jak je naznačeno výše, interakce obou gasotransmiterů (NO a H_2S) mohou vést k formování metabolitů s antioxidačním účinkem (e.g. HNO), nicméně antioxidační vlastnosti byly pozorovány i u samotných gasotransmiterů, které tak mohou posílit antioxidační mechanismy přítomné ve spermii.

2.7 Antioxidační systémy spermie

Zatímco spermie se svým charakteristicky nízkým obsahem cytoplazmy mají velmi limitované zdroje antioxidační ochrany, semenná plazma představuje hlavní zdroj antioxidantů chránících samčí gamety před oxidačním stresem. Mezi hlavními antioxidanty semenné plasmy lze uvést SOD a katalázu, jejichž efekt je podpořen dalšími substancemi podobného účinku, jako je například albumin, glutation, pyruvát turín či vitamin E a C (de Lamirande et Gagnon, 1995). Z uvedených látek s antioxidačními vlastnostmi lze odvodit dvě základní skupiny: enzymatické antioxidanty, které lze označit za přirozené a dále syntetické antioxidanty, jejichž koncentrace je závislá na jejich příjmu z potravy. Tělu vlastní, enzymatické antioxidanty, mají schopnost narušit oxidační řetězec, a tak snížit negativní dopad oxidačního stresu. V konkrétním příkladu, SOD vychytává superoxidové anionty, které redukuje na méně reaktivní peroxid vodíku, který je dále zpracován katalázou na kyslík a vodu. Peroxid vodíku je také redukován na vodu a alkohol skrze

aktivitu glutation peroxidázy (GPx), kterou doplňuje glutation reduktáza (GR). Společně GPx a GR chrání buňky před lipidovou peroxidací udržováním fyziologické hladiny glutationu a jeho oxidované formy (H. Funahashi et Sano, 2005; Matoušková et al., n.d.). Hladina syntetických antioxidantů je závislá na příjmu vitamínů, peptidů a minerálů v potravě, jako je tomu například u glutationu, jenž slouží jako kofaktor k činnosti selen-dependentní GPx. Jedná se o jeden z nejefektivnějších prostředků ochrany před oxidačním stresem u býčích a kančích spermií (Funahashi et Sano, 2005). Jako prekurzor pro biosyntézu glutationu slouží cystein, který tak může nepřímo napomáhat ochraně membránových lipidů a proteinu před volnými radikály. Navíc cystein vykazuje kryoprotektivní účinek, jelikož napomáhá k zachování funkční integrity akrozomu a mitochondrií a tím napomáhá ke zvýšení viability a motility. Tento efekt byl pozorován také v případě zchlazených kančích dívek (Bansal et Bilaspuri, 2011). Zvýšený obsah cysteinu v ředidle kančích inseminačních dávek má pozitivní efekt na viabilitu spermií a jejich schopnost penetrace oocytu. Běžně využívaný syntetický antioxidant, který je součástí většiny ředících medií pro kančí spermie, představuje hovězí sérový albumin (BSA) (Bansal et Bilaspuri, 2011). Ve spermii také hrají důležitou roli při redukci oxidačního stresu peroxiredoxiny a thioredoxiny krom výše zmíněních antioxidantů (O'Flaherty, 2014).

2.7.1 Antioxidační vlastnosti NO a H_2S

Účinek NO a H_2S ve vztahu k antioxidačním aparátu spermie se odvíjí od jejich koncentrace. Jak bylo zmíněno výše, nadměrná akumulace těchto dvou gasotransmiterů vede ke zvýšení míry oxidačního stresu a narušení oxidační rovnováhy, avšak v rámci fyziologických koncentrací mohou obě látky podpořit antioxidační status spermií, či antioxidační vlastnosti semenné plazmy.

NO a Oxidační Stres

Na jednu stranu jeden ze tří gasotransmiterů, NO představuje zároveň výchozí sloučeninu pro tvorbu RSS, kdy reaguje s kyslíkem a superoxidem a vytváří sloučeniny jako je dioxid dusičitý, oxid dusitý a další (Otasevic et al., 2020). Avšak často je opomíjen fakt, že NO může také narušit oxidační řetězec, kyd reaguje s lipidovými peroxylovými radikály a vykazuje tak i antioxidační účinky (Radi, 2018). Převážná většina studií byla zaměřena na oxidativní vlastnosti NO vedoucí k poškození membrány, či narušení integrity DNA a snížení mitochondriální aktivity (Weidinger et Kozlov, 2015). Na rozdíl od konstitutivních forem NOS, které zajišťují produkci NO nezbytnou k buněčné signalizaci, iNOS je isoforma odpovědná za produkci nadměrného množství NO, které slouží k zneškodnění patogenů (Wolff et Lubeskie, 1995). Proto byla publikována řada studií zkoumající možnosti inhibice jednotlivých isoforem NOS (Alizadeh et al., 2010; Bahmanzadeh et al., 2008). V centru pozornosti přitom byla iNOS, jejíž aktivita je typická pro zánětlivé reakce a bývá spojena

s běžnými disfunkcemi samčí reprodukční soustavy (Alizadeh et al., 2010). Blokování iNOS tedy přispívá k udržení fyziologické hranice NO.

Antioxidační vlastnosti vykazují i sloučeniny guanidinu, mezi které patří i aminoguanidin (AG), což je sloučenina hydrazinu a L-argininu rozpustná ve vodě a je využívána jako selektivní inhibitor iNOS (Misko et al., 1993). Řada studií již zkoumala možnosti tohoto selektivního inhibitoru iNOS ve vztahu k léčbě diabetu, rakoviny, či infarktu (Corbett et al., 1992; Courderot-Masuyer et al., 1999; Janakiram et Rao, 2012; Sun et al., 2010). Naopak možností využití AG při řešení reprodukčních poruch se zabývalo relativně málo studií (Abbasi et al., 2011; Alizadeh et al., 2016; Oguz et al., 2013). Yildiz et al. (1998) ve své studii potvrdili schopnost AG vychytávat peroxynitrite a ROS, na což navázali Courderot-Masuyer et al. (1999), kteří prokázali schopnost AG vychytávat hydroxylové a peroxylové radikály. Zároveň byla u AG prokázána schopnost snižovat míru lipidové peroxidace, přičemž antioxidační schopnosti se odvíjí od koncentrace AG (Philis-Tsimikas et al., 1995).

H_2S a oxidační stres

Stejně jako předchozí gasotransmiter i H_2S lze řadit mezi reaktivní sloučeniny. Stojí na počátku vzniku celé řady reaktivních sloučenin síry (RSS) (Mishanina et al., 2015). RSS lze definovat jako látky, které obsahují alespoň jeden redoxně aktivní atom síry, či funkční skupinu obsahující síru a zároveň jsou schopné vstoupit do redoxních reakcí s biomolekulami při fyziologických podmínkách za účelem spuštění či šíření buněčného signálu (Giles et al., 2017). Stejně jako v případě ROS a RNS, i RSS jsou látky nezbytné pro fyziologické pochody v buňce avšak jejich nadměrná akumulace vede k oxidačnímu stresu a poškození buněčných komponent (Otasevic et al., 2020).

Wang et al. (2018) ve své studii zaměřené na astenozoospermické muže pozorovali negativní vliv 5 µM NaHS na motilitu spermií, což přisuzovali uvolnění nadměrného množství sulfanu. K obdobnému závěru došli Zhao et al. (2016), kdy podávání Na_2S v in vitro podmínkách (25-100 µM) a in vivo podmínkách (10 mg/kg tělesné váhy) vedlo ke snížení motility kančích a myších spermií, v tomto pořadí. Snížení produkce ATP mitochondriemi se nabízí jako potenciální vysvětlení pro snížení motility spermií po aplikaci donorů NaHS ve vyších koncentracích. Již v několika buněčných typech byla pozorována inhibice mitochondriálního IV komplexu za použití NaHS při koncentraci 10 µM a vyšší (Szabo et al., 2014). Zvýšení oxidačního stresu bylo pozorováno u kančích spermií při použití vysoké koncentrace donoru H_2S (50 µM Na_2S) (Zhao et al., 2016).

Na druhou stranu byly pozorovány i antioxidační účinky H_2S, který zároveň působí jako redukční činidlo (Li et al., 2011). Při nízkých koncentracích může H_2S a jeho disociovaná forma HS^- přímo vychytávat jak ROS tak RNS (e.g. O_2, H_2O_2, peroxynitrit) (Shefa et al., 2018). Na druhou

stranu přímé vychytávání RS se zdá být málo pravděpodobné v porovnání s jinými antioxidanty vzhledem k velmi nízkým fyziologickým koncentracím H_2S v buňce (Shefa et al., 2018; Xie et al., 2016). V tomto smyslu je pravděpodobnější nepřímé navýšení antioxidační kapacity buňky. U testikulárních zárodečných buněk vystavených tepelnému stresu byla pozorována zvýšená aktivita SOD a snížená hladina ROS po aplikaci NaHS (Li et al., 2015). Zároveň Li et al. (2015) pozorovali rekuperaci dysfunkčních mitochondrií se sníženou produkcí ATP a zvýšenou spotřebou O_2, což naznačuje, že H_2S by mohl bránit buněčné apoptóze. V souhlasu s předcházející studií Ning et al. (2018) pozorovali zvýšenou expresi SOD a snížení počtu apoptotických buněk u testikulárních buněk vystavených tepelnému stresu po aplikaci donoru H_2S (GYY4137). Autoři zároveň změřili expresi mitochondriálních apoptotických proteinů: Bax, Bcl-2 a kaspázy 3. Po aplikaci donoru H_2S se vyrovnal poměr proteinů Bax a Bcl-2 (Ning et al., 2018). Právě poměr těchto dvou proteinů podmiňuje aktivaci apoptózy (Jia et al., 2009). Právě udržením správného poměru Bax a Bcl-2 došlo ke snížené expresi kaspázy 3 po aplikaci GYY4137 (Ning et al., 2018). Aktivace kaspázy 3 spouští signální kaskádu, kdy už nejde zvrátit proces apoptózy (Porter et Jänicke, 1999). Zdá se tedy, že H_2S může vykazovat jak přímé antioxidační vlastnosti a stejně tak může i nepřímo navýšit antioxidační mechanismy v buňce.

Antioxidační vlastnosti gasotransmiterů jsou zajímavé z hlediska prevence zvyšování koncentrace ROS během in vitro manipulace se samčími gametami (Rodriguez et al., 2017). Vzniklý oxidační stres vede v případě savčích spermií k lipidové peroxidaci, která představuje jednu z hlavních příčin snížené motility, která má negativní dopad na fertilizační potenciál inseminačních dávek (Aitken, 2017). Zároveň však není žádoucí přílišná koncentrace antioxidantů v inseminační dávce, jelikož by snížila koncentraci RS pod fyziologicky nezbytnou úroveň a tím omezila klíčové funkční vlastnosti spermií (Sikka, 2004). Proto je třeba při hodnocení efektu antioxidantů na kvalitu spermií brát v úvahu nejen přímé ukazatele, jako je antioxidační kapacita, ale také nepřímé ukazatele, které představují právě funkční vlastnosti spermií, kde lze mezi hlavními parametry uvést motilitu a integritu cytoplazmatické membrány a akrozomu.

Část přehledu literatury byla publikována v review: **Kadlec**, M., Ros-Santaella, J. L., Pintus, E. 2020. The Roles of NO and H2S in Sperm Biology: Recent Advances and New Perspectives. International Journal of Molecular Sciences. 21 (6). 2174., které je součástí příloh.

3 Hypotéza a cíle práce

Byla stanovena hypotéza, že gasotransmitery mohou zlepšovat funkční vlastnosti kančích spermií a ovlivňovat míru jejich oxidativního stresu:

1. Selektivní inhibitor iNOS, aminoguanidin, zmírní dopady oxidačního stresu na kančí spermie.
2. Efekt sulfanu na kančí spermie se bude lišit v závislosti na použitém donoru.
3. Interakcí mezi oxidem dusnatým a sulfanem lze posílit positivní efekt obou gasotransmiterů na kančí spermie v přítomnosti oxidačního stresu.

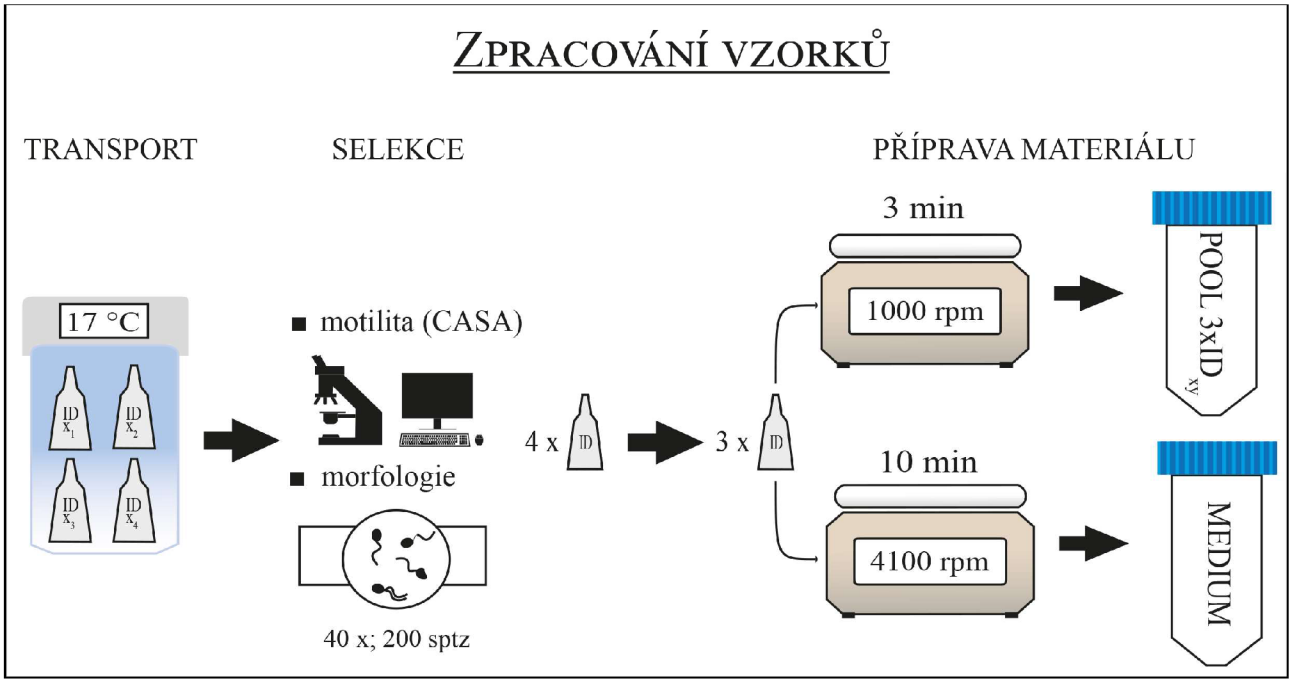
Pro ověření stanovené hypotézy byly stanoveny následující cíle:

1. Ověřit vliv aminoguanidinu na funkční vlastnosti a markery oxidačního stresu u kančích spermií vystavených oxidačním stresu.
2. Porovnat efekt rozdílných donorů sulfanu na funkční vlastnosti a markery oxidačního stresu u kančích spermií vystavených oxidačnímu stresu.
3. Ověřit interakci donoru NO s donorem H_2S v přítomnosti oxidačního stresu porovnáním funkčních vlastností a markerů oxidačního stresu u vzorků s jedním donorem se vzorky s dvěma donory.

4 Materiály a metody

K experimentům byly použity komerční inseminační dávky, které byly dopraveny do laboratoře a skladovány při 17 °C v chladícím boxu až do jejich zpracování. V laboratoři byla vyhodnocena kvalita každé inseminačních dávky pomocí počítačem řízené analýzy spermií (CASA) a morfologie spermií. Inseminační dávky s nejlepšími parametry se centrifugovaly (1000 rpm/3 min. / 167 g) a odebral se supernatant zbavený části defektních spermií. Pro práci se smíchaly inseminační dávky tří rozdílných kanců v poměru 1:1:1. Následný směsný vzorek se ředil na vhodnou finální koncentraci (20 x 10^6/ml). Pro naředění se používalo médium extrahované z inseminační dávky pomocí centrifugace (4100 rpm/10 min.; Obr.3).

Všechny chemikálie byly pořízeny od firmy Sigma-Aldrich (Česká republika), pokud není uvedeno jinak. Pro ověření prvního cíle se využil aminoguanidin hydrochlorid. Pro ověření efektu sulfanu na spermie se požilo rychlého donoru Na_2S (Na^2S × 9 H_2O) a pomalého donoru GYY4317 (C11H16NO2PS2·C4H9NO × CH2Cl2) sulfanu. Cílová koncentrace donoru se stanovila na základě dostupné literatury a vzhledem výsledkům přípravných experimentů (nepublikovaná data). Vliv oxidu dusnatého na spermie se ověřil pomocí nitroprusidu sodného (SNP). Při zkoumání interakce NO a H_2S se použil rychlý donor sulfanu NaHS a donoru oxidu dusnatého SNP. Indukce oxidačního stresu byla navozena aplikací systému askorbát sodný/sulfát železitý (Fe^{2+}/askorbát) ve vodném roztoku, který se přidal do zkoumaných vzorků o koncentraci dle dostupné literatury. Tento systém je vhodný pro navození lipidové peroxidace. Vzorky se po dobu celou dobu trvání experimentů inkubovaly ve vodní lázni při 38°C. Ve stanovené časy se hodnotily níže zmíněné parametry.



Obrázek 3. Postup zpracování komerčních inseminačních dávek (Kadlec, 2022).

Klíčové odlišnosti v experimentálním designu jednotlivých pokusů jsou popsány zvlášť pro každý experiment. Společné metody kvalitativního hodnocení spermií a markerů oxidačního stresu zahrnovaly:

1) Stanovení morfologie

Spermie se ředily pomocí PBS bufferu a fixovaly 2,5% glutaraldehydem. Následně se hodnotila suspenze světelnou mikroskopií při 40x zvětšení.

2) Počítačem řízená analýza (CASA)

Analýza zahrnovala stanovení standartních parametrů: celkové motility (% motilních spermií), progresivní motility (% spermií s minimální VAP), linearita (LIN), přímost (STR), amplituda laterárního vybočení hlavičky spermie (ALH), frekvence křížení (BCF), rychlost křivočarého pohybu (VCL), rychlost přímého pohybu (VSL) a rychlost po průměrné trajektorii (VAP).

Systém CASA se skládal, krom stolního počítače, z mikroskopu Eclipse E600 (Nikon, Tokyo, Japonsko), který byl vybaven objektivem s 10x negativním fázovým kontrastem (Nikon, Tokyo, Japonsko), výhřevnou deskou (38 ˚C; Tokai Hit, Shizuoka, Japonsko), a DMK 23UM021 digitální kamerou (The Imaging Source, Bremen, Německo). Rychlost záznamu byla stanovena na 60 obrázků za vteřinu (60 FPS), minimálně zaznamenáno 31 obrázků. Celkem bylo minimálně hodnoceno 200 spermií.

3) Analýza integrity plazmatické membrány (PI-CFDA)

Vzorek byl zředěn v PBS bufferu, který obsahoval fluorescein jodid propidia (PI; (zásobní roztok obsahoval 0.5 mg/mL PI v PBS), karboxyfluorescein diacetát (CFDA; zásobní roztok obsahoval 0.46 mg/mL CFDA v dimetyl sulfoxidu) a 0,3% formaldehyd. Vzorky se následně inkubovaly 10 min. při 38 °C a poté se hodnotila pod epifluorescenčním mikroskopem při 40x zvětšení. Spermie s neporušenou membránou byly zbarveny zeleně (CFDA), spermie s poškozenou membránou byly zbarveny červeně (jodid propidia). Počítá se 200 spermií.

4) Analýza integrity akrozomu (PNA-FITC)

Připravil se roztěr vzorku a fixoval se v methanolu po dobu 10 minut. Následně se vzorky promývaly v PBS. Roztěry se inkubovaly v temnotě při 38 °C po dobu 10 minut v přítomnosti

aglutininu podzemnice olejné (PNA) konjugovaného s fluorescein-5-isothiokyanátem (FITC) v roztoku PBS. Inkubace probíhala ve vlhké komoře. Následně se vzorky promývaly v PBS a hodnotily se pomocí epifluorescence při 40x zvětšení. V každém vzorku bylo vyhodnoceno 200 spermií, zeleně zbarvené spermie měly intaktní akrozom.

5) Analýza integrity akrozomálního hřebene (NAR test)

Odebraný vzorek se fixoval 2,5% glutaraldehydem a byl vyhodnocen pomocí fázového kontrastu při 40x zvětšení. V každém vzorku bylo hodnoceno 200 spermií.

6) Analýza lipidové peroxidace plazmatické membrány

Lipidová peroxidace se stanovila pomocí kyseliny thiobarbiturové (TBARS). Při stanoveném času byly odebrány vzorky a skladovány při -80 °C až do analýzy. Měřila se absorbance každého vzorku při vlnové délce 532 nm pomocí spektrofotometru. Referenční křivka byla stanovena pomocí 1,1,3,3-tetrametoxypropanu (MDA) o známé koncentraci. Míra lipidové koncentrace byla vyjádřena jako µmol MDA na 10^8 spermií. Každý vzorek byl měřen dvakrát.

7) Analýza celkové antioxidační kapacity (TAC)

Ve stanovený čas se odebral vzorek, který byl centrifugován po 10 min. při 2000 g a 4 °C a následně byl supernatant odebrán a uložen do -80 °C až do vlastní analýzy. Celková antioxidační kapacita se stanovila pomocí spektrofotometru při vlnové délce 660 nm za využití 2-azinobis-(3-etylbenzotiazoline-6-sulfoniové kyseliny (ABTS). Referenční křivka se stanovila pomocí 6-hydroxy-2,5,7,8-tetrametylchroman-2-karboxylové kyseliny (Trolox). Antioxidační kapacita byla vyjádřena jako ekvivalent Troloxu v mM.

Design jednotlivých experimentů a specifické parametry hodnocení:

4.1 Ověření vlivu aminoguanidinu na funkční vlastnosti a markery oxidačního stresu u kančích spermií vystavených oxidačním stresu

Experimentální skupin a doba inkubace: Ctr, Ctr-OX, Ag 0,1 mM, 1mM, 10 mM; 20 min (pouze Ctr), 120 min, 210 min

Příprava vzorků a koncentrace donorů: Byl indukován oxidační stres pomocí Fe^{2+}/askorbátu sodného (0,5 mM/ 0,5 mM v PBS) u následujících vzorků: Ctr-OX, Ag 0,1 mM, 1mM, 10 mM; aminoguanidin byl ředěn v PBS pro připravení zásobního 0,2 M roztoku.

Markery oxidačního stresu: TAC, lipidová peroxidace

Funkční vlastnosti spermií: index motility spermií (SMI), CASA, integrita plazmatické membrány (HOST, CFDA-PI), integrita akrozomu (NAR test, PNA-FITC)

SMI: Byla stanovena subjektivní kvalita pohybu (QM) na stupnici 0-5 (0 pro žádný pohyb, 5 pro intenzivní progresivní pohyb). Dále bylo subjektivně stanoveno procento motilních spermií zaokrouhlené na nejbližších 5 %. Získané hodnoty byly převedeny na SMI pomocí formule: [SM + (QM x 20)]/2.

HOS test (test hypoosmotické bobtnavosti): Byl připravený hypoosmotický roztok (7,35 g/l citrát sodný a 13,51 g/l fruktóza) temperovaný na 38 °C. Vzorky byly inkubovány v hypoosmotickém roztoku po dobu 30 minut. Počítáno bylo 200 spermií pomocí 40x objektivu s fázovým kontrastem. Spermie se stočeným bičíkem byly hodnoceny jako spermie s nepoškozenou plazmatickou membránou bičíku.

Statistická analýza: Statistická analýza byla provedena za využití softwaru SPSS 20.0 (IBM Inc., Chicago, IL, USA). Pro kontrolu normality distribuce byl využit Shapiro-Wilkův test. Pro porovnání kontrolního vzorku (Ctr) v rozdílných časech inkubace byly použity ANOVA a Friedmanův test v závislosti na normalitě rozložení dat. Generalizované linearní modely byly použity k vzájemnému porovnání vzorků v daných časech. Data byla vyjádřena jako průměr ± směrodatná odchylka. Statistická hladina významnosti p byla 0,05.

4.2 Porovnání efektu rozdílných donorů sulfanu na funkční vlastnosti a markery oxidačního stresu u kančích spermií vystavených oxidačnímu stresu

Experiment I. Stanovení antioxidačních vlastností a stability pomalého a rychlého donoru sulfanu (Na_2S a GYY4317)

Experimentální skupiny a doba inkubace: Na_2S (150, 300, 600, 1200, 2400 μM), GYY4317 (150, 300, 600, 1200, 2400 μM); 20 min, 120 min, 210 min

Příprava vzorků a koncentrace donorů: Na_2S / GYY4137 byly ředěny v roztoku PBS do finální koncentrace 2,400, 1,200, 600, 300, a 150 μM.

Hodnocení antioxidačních schopností: TAC

Statistická analýza: Pro ověření normality distribuce byl použit Shapiro-Vilkův test a pro ověření homogenity dat byl použit Levenův test. Mann-Whitney U-test byl využit pro porovnání TAC Na_2S a GYY4317 o stejné koncentraci. Friedmanův test opakovaných měření byl použit pro porovnání

TAC obou donorů H_2S v průběhu inkubace. Data byla vyjádřena jako průměr ± směrodatná odchylka. Statistická hladina významnosti *p* byla 0,05.

Experiment II. Ověření účinku donorů sulfanu na spermie vystavené oxidačnímu stresu.

Experimentální skupiny a doba inkubace: Ctr, Ctr-OX, Na_2S (3, 30, 300 μM), GYY4317 (3, 30, 300 μM); 20 min (pouze Ctr) a 210 min

Příprava vzorků a koncentrace donorů: Byl indukován oxidační stres pomocí Fe^{2+}/askorbátu sodného (0,1 mM/ 0,5 mM v PBS) v následujících vzorcích: Ctr-OX, Na_2S (3-300 μM), GYY4317 (3-300 μM); donory sulfanu byly naředěny v PBS na požadovanou koncentraci.

Markery oxidačního stresu: lipidová peroxidace

Funkční vlastnosti: CASA, integrita plazmatické membrány (CFDA-PI), integrita akrozomu (NAR test, PNA-FITC), mitochondriální aktivita (rhodamin 123-PI)

Analýza mitochondriální aktivity: Vzorky byly inkubování v přítomnosti rhodaminu 123 (5 mg/ml, w/v, v dimetylsulfoxidu, DMSO) a propidium iodidu (0,5 mg/ml, w/v, v PBS) po dobu 15 minut při 38 °C v temné komoře. Po inkubaci byly vzorky centrifugovány při 500 g po dobu 5 minut. Po odsátí supernatantu byl vzorek resuspendován v PBS. Hodnoceno bylo 200 spermií pomocí epifluorescenčním mikroskopie při 40x zvětšení. Spermie vykazující intenzivní světle zelenou fluorescenci v proximální části bičíku se považovaly za spermie s aktivními mitochondriemi.

Statistická analýza: Pro ověření normality distribuce byl použit Shapiro-Vilkův test a pro ověření homogenity dat byl použit Levenův test. Pro porovnání efektu a typu donoru na variabilní parametry spermii byly použity generalizované lineární modely (GZLM). Data byla vyjádřena jako průměr ± směrodatná odchylka. Statistická hladina významnosti *p* byla 0,05.

4.3 Ověření interakce donoru NO s donorem H_2S v přítomnosti oxidačního stresu porovnáním funkčních vlastností a markerů oxidačního stresu u vzorků s jedním donorem se vzorky s dvěma donory

Experimentální skupiny a doba inkubace: Ctr, Ctr-OX, DD (0,1 μM SNP + 0,1 μM NaHS), SNP (0,1 μM), NaHS (0,1 μM); 20 min (pouze Ctr) a 90 min

Příprava vzorků a koncentrace donorů: Byl indukován oxidační stres pomocí Fe^{2+}/askorbátu sodného (0,5 mM/ 0,5 mM v PBS) u následujících vzorků: Ctr-OX, DD, SNP, NaHS; donory gasotransmiterů (SNP/NaHS) byly naředěny v PBS na požadovanou koncentraci.

Markery oxidačního stresu: TAC, lipidová peroxidace

Funkční vlastnosti: CASA, integrita plazmatické membrány (CFDA-PI), integrita akrozomu (PNA-FITC)

Statistika: Normalita distribuce byla analyzována pomocí Shapiro-Vilkova testu. Pro analýzu vlivu jednotlivých donorů a jejich kombinace na pozorované parametry byly použity generalizované linearní modely (GZLM). Data byla vyjádřena jako průměr ± směrodatná odchylka. Statistická hladina významnosti p byla 0,05.

Analýza subpopulací na základě motility: Počet subpopulací byl stanoven automaticky pomocí dvojstupňového shlukování za využití Eukleidovské vzdálenosti a Schwartz-Bayesova kritéria. Získaný počet shluků byl použit k analýze nejbližších středů (K-means cluster test) daných shluků. Kruskalův-Wallisův test byl použit k ověření rozdílů mezi subpopulacemi spermií jednotlivých vzorků. K ověření odlišnosti kinetických parametrů jednotlivých subpopulací byl použit Wilcoxův párový test. Data byla vyjádřena jako průměr ± směrodatná odchylka. Statistická hladina významnosti p byla 0,05.

5 Výsledky a diskuse

5.1 Vliv aminoguanidinu na funkční vlastnosti a markery oxidačního stresu u kančích spermií vystavených oxidačním stresu

Endogenní produkci NO zajišťuje NOS, která se vyskytuje ve třech isoformách (Förstermann et Kleinert, 1995). Všechny tři isoformy NOS se podílejí na klíčových reprodukčních procesech (Dixit et Parvizi, 2001), a jsou exprimovány v lidských, myších i kančích spermiích (Aquila et al., 2011; Herrero et al., 1997; Herrero et al., 1996). Avšak inducibilní isoforma, iNOS, je spojována především s aktivitou buněk imunitního systému během infekce, či zánětlivých stavů. Leukocyty přítomné v semenné plazmě představují jeden z hlavní zdrojů ROS (Gomez et al., 2021; Kessopoulou et al., 1992). V této studii byl testován aminoguanidin, který představuje selektivní inhibitor iNOS a zároveň působí jako antioxidant (Abbasi et al., 2011).

Výsledky studie jasné prokázaly silný antioxidační potenciál aminoguanidinu, který také zachovává motilitu spermií, snižuje míru lipidové peroxidace a chrání plazmatickou membránu a integritu akrozomu u kančích spermií vystavených indukovanému oxidačnímu stresu. Zajímavé hodnoty vykazoval vzorek s 1 mM koncentrací aminoguanidinu, který se nelišil, či dokonce vykazoval lepší výsledky z hlediska funkčních parametrů spermií, než kontrolní vzorek bez indukovaného oxidativního stresu (Ctr), což dokazuje, že negativní dopad oxidativního stresu byl zcela odstraněn. Nehledě na dobu inkubace, u vzorků s koncentrací aminoguanidinu 1 a 10 mM byla pozorována vyšší antioxidační kapacita, než u kontrolních vzorků (Ctr a Ctr-OX). Zároveň nebyl skrze hodnocené parametry odhalen cytotoxický efekt.

Vzhledem k výsledkům naší studie se zdá být aminoguanidin vhodným činitelem k potlačení negativního dopadu oxidačního stresu, což odpovídá závěrům dalších studií zaměřených na další typy buněk a tkání (Abo-Salem, 2012; Abraham et Rabi, 2011; Eroglu et al., 2008). Celková antioxidační kapacita u vzorku s 1mM aminoguanidinem odpovídala fyziologické antioxidační kapacitě kančí semenné plazmy (Barranco et al., 2015; Zakošek Pipan et al., 2014). Vyšší antioxidační kapacita semenné plazmy chrání spermie při zpracování v in vitro podmínkách (e.g. výroba inseminačních dávek), což vede k lepší reprodukčním výsledkům (e.g. velikost vrhu)(Barranco et al., 2015). V této studii byl pozorován pozitivní vliv aminoguanidinu na klíčové parametry spermií (integrita plazmatické membrány a akrozomu a motilita), které jsou spojovány s fertilizačním potenciálem hospodářských zvířat (Jung et al., 2015; Kastelic et Thundathil, 2008; Love, 2016). I když spermie ve vzorku s 10 mM aminoguanidinem vykazovaly více než dvakrát vyšší motilitu než kontrolní vzorek vystavený oxidačnímu stresu, progresivní motilita a lineární motilita byly značně snížené. Pravděpodobné vysvětlení nabízí značně zvýšená antioxidační kapacita v porovnání s fyziologickou

antioxidační kapacitou (Barranco et al., 2015; Zakošek Pipan et al., 2014). Zdá se, že vysoká koncentrace aminoguanidinu snížila hladinu ROS pod fyziologicky nezbytnou úroveň, což vedlo k částečnému snížení zmíněných parametrů motility, aniž by to mělo negativní vliv na další parametry spermií. Aminoguanidin ochránil akrozom před negativním dopadem oxidačního stresu, přičemž integrita akrozomu je zásadní pro správný průběh akrozomální reakce a průnik spermie ochrannými vrstvami oocytu (Bonet et al., 2013). Částečná akrozomální reakce, ke které dochází během inkubace či ve fertilizačním médiu, je hlavní příčinou polyspermie v asistované reprodukci prasat (Hiroaki Funahashi, 2003). V tomto smyslu lze prohlásit, že aminoguanidin zvyšuje fertilizační potenciál skrze ochranu integrity akrozomu.

Výsledky studie tedy prokázaly antioxidační účinky selektivního inhibitoru iNOS, aminoguanidinu, který zároveň napomáhá k udržení funkčních vlastností kančích spermií v přítomnosti oxidačního stresu. Aminoguanidin se zdá být vhodným prostředkem k prevenci snižování kvality ejakulátu v důsledku narůstající koncentrace ROS v průběhu skladování.

Uvedené výsledky jsou součástí publikace (viz Přílohy):

Pintus, E., **Kadlec**, M., Jovičić, M., Sedmíková, M., Ros-Santaella, J. L. 2018. Aminoguanidine protects boar spermatozoa against the deleterious effects of oxidative stress. Pharmaceutics. 10 (4). doi: 10.3390/pharmaceutics10040212.

5.2 Efekt rozdílných donorů sulfanu na funkční vlastnosti a markery oxidačního stresu u kančích spermií vystavených oxidačnímu stresu

U sulfanu byl prokázán jak oxidační, tak antioxidační efekt. Wang et al. (2018) pozorovali sníženou hladinu H_2S v semenné plazmě u mužů s astenozoospermií a suplementací H_2S dosáhli zlepšení motility. Naopak u kančích spermií byl pozorován negativní dopad H_2S na motilitu, viabilitu a mitochondriální aktivitu (Zhao et al., 2016). Za tímto rozporem může stát i divergentní povaha donorů H_2S, jejichž efekt se odvíjí od koncentrace a způsobu uvolňování H_2S. Primárně se využívá anorganických solí NaHS a Na_2S, které se označují jako rychlé donory sulfanu, jelikož uvolňují velké množství sulfanu v krátkém časovém intervalu (Rose et al., 2015). Dále byly vyvinuty syntetické donory H_2S, mezi které se řadí i GYY4137 (derivát fosforoditiolátu) použitý v této studii, které uvolňují menší množství sulfanu během delšího časového intervalu, čímž se napodobuje fyziologická produkce tohoto gasotransmiteru. V této studii byly porovnány antioxidační vlastnosti a účinek rychlého (Na_2S) a pomalého (GYY4137) donoru sulfanu v přítomnosti oxidačního stresu.

V první části experimentu byly poprvé popsány rozdíly v antioxidačních schopnostech dvou donorů sulfanu, Na_2S a GYY4137, při standardizovaných podmínkách (fyziologické pH, 38 °C) v průběhu inkubace (20, 120 a 210 minut). Výsledky prokázaly, že antioxidační schopnost Na_2S je méně stabilní v porovnání s GYY4317, jehož antioxidační kapacita má vzrůstající tendenci v průběhu inkubace. Zároveň u GYY4317 byla pozorována větší antioxidační kapacita než u Na_2S, nehledě na dobu inkubace.

V druhé části experimentu aplikace GYY4137 o koncentraci 3 a 30 µM vedla k zachování motility v porovnání s kontrolním vzorkem s indukovaným oxidačním stresem. Zároveň nebyl pozorován cytotoxický efekt GYY4137 nehledě na použitou koncentraci. U vzorků s 3 a 30 µM GYY4317 byla pozorována motilita spermií srovnatelná s kontrolním vzorkem bez indukovaného oxidačního stresu. Navíc u vzorku s 30 µM GYY4317 bylo pozorováno vyšší zastoupení spermií s progresivní motilitou než u kontrolního vzorku s indukovaným oxidačním stresem (Ctr-OX). Zatímco při nejnižší 3 µM koncentraci Na_2S pomohl zachovat motilitu spermií v porovnání s Ctr-OX, při nejvyšší 300µM koncentraci měl jednoznačně cytotoxický efekt. Zajímavý účinek byl pozorován u vzorku s 30 µM Na_2S, kde nebyly pozorovány změny v mitochondriální aktivitě, integritě plazmatické membrány a akrozomu, či lipidové peroxidaci, avšak došlo ke značnému snížení motility.

Částečné zmírnění negativního dopadu oxidačního stresu na spermie pomocí donorů sulfanu v této studii bylo pozorováno i v případě dalších typů buněk (Kimura et al., 2010; Yonezawa et al., 2007). V době publikování článku bylo dostupných pouze několik studií zaměřených na porovnání účinku pomalých a rychlých donorů sulfanu u buněk vystavených oxidačním stresu (Cao et al., 2018;

Wang et al., 2018; Whiteman et al., 2010). V souhlasu s těmito studiemi naše výsledky ukázaly, že efekt donorů H_2S se odvíjí od dávky a typu donoru. Avšak tato studie je první, která zkoumá působení pomalého a rychlého donoru sulfanu na spermie vystavené oxidačnímu stresu. V porovnání s GYY4317, efekt Na_2S byl značně ovlivněn jeho koncentrací. Tento vzorec účinku odpovídá dvojfázové křivce biologické odezvy na vzrůstající koncentraci sulfanu: v malých koncentracích vykazuje antioxidační účinek a ve vyšších koncentracích působí jako pro-oxidační činidlo (Szabo et al., 2014). Ke stejnému závěru došli i Zhao et al. (2016), kdy Na_2S při koncentraci 25 µM omezil motilitu spermií, aniž by ovlivnil jejich viabilitu a membránový potenciál mitochondrií (Zhao et al., 2016). Stejně tak i rychlý donor NaHS snižuje motilitu myších a lidských spermií (Wang et al., 2018; Wiliński et al., 2015). Zajímavé zdůvodnění nabízí i inhibiční účinek sulfanu na IV komplex mitochondriálního řetězce (cytochrom c oxidázu), což vede k omezení produkce ATP (Szabo et al., 2014). U savčích spermií je většina ATP generovaného v mitochondriích určena k zachování pohybu (Storey, 2008), snížením ATP dochází k omezení motility. Stejně tak v této studii vzorky s 300 µM Na_2S neobsahovaly žádné spermie s aktivními mitochondriemi. Tento jev lze přisuzovat zvýšené produkci ROS po aplikaci Na_2S (Zhao et al., 2016), což odpovídá i zvýšené lipidové peroxidaci a snížené integritě plazmatické membrány a akrozomu pozorované v této studii.

Publikovaná práce popsala antioxidační vlastnosti dvou donorů sulfanu, Na_2S a GYY4317 při fyziologických podmínkách, což může posloužit dalším studiím zaměřených na buněčnou fyziologii. Z výsledků práce jasně vyplývá, že možnosti využití sulfanu při mírnění dopadu oxidačního stresu na funkční vlastnosti kančích spermií se odvíjí od typu a koncentrace použitého donoru.

Uvedené výsledky jsou součástí publikace (viz Přílohy):

Pintus, E., Jovičić, M., **Kadlec**, M., Ros-Santaella, J. L. 2020. Divergent effect of fast- and slow-releasing H2S donors on boar spermatozoa under oxidative stress. Scientific Reports. 10 (1). 6508. doi: 10.1038/s41598-020-63489-4.

5.3 Porovnání funkčních vlastností kančích spermií a markerů oxidačního stresu u vzorků s jedním donorem gasotransmiteru se vzorky s donory obou gasotransmiterů

Kromě koncentrace může mít vliv na účinek gasotransmiteru také přítomnost dalšího gasotransmiteru. U somatických buněk byla prokázána interakce oxidu dusnatého (NO) a sulfanu (H_2S), zatímco v případě studia samčích gamet byla zkoumána pouze akce jednotlivých donorů NO a H_2S zvláště. V těchto studiích bylo prokázáno, že při nízkých koncentracích vykazují oba gasotransmitery positivní efekt na kvalitu spermií. Zároveň efekt jednotlivého gasotransmiteru může být ovlivněn přítomností oxidačního stresu, který je spojován s relativně častými poruchami reprodukce samců. V této studii byl porovnán efekt kombinace donorů SNP a NaHS (donor NO a H_2S v tomto pořadí) s efektem jejich individuální aplikace na kančí spermie vystavené oxidačnímu stresu.

U vzorků obsahujících jednotlivé donory a jejich kombinaci byly pozorovány zajímavé rozdíly v progresivní motilitě spermií a integritě plazmatické membrány (PMI). Vzorek obsahující kombinaci obou donorů (DD) vykazoval signifikantně vyšší PMI a PMot, než CtrOX po 90 minutách inkubace a zároveň se integrita plazmatické membrány nelišila od Ctr vzorku po dvaceti minutách inkubace. Dále PMot spermií v DD vzorku překonal PMot vzorku s NaHS. U vzorku SNP byl pozorován mírný účinek na TMot a PMot, kdy se pozorované hodnoty nacházely mezi hodnotami vzorku NaHS a DD. Vzorek s SNP byl charakterizován vyšší směrodatnou odchylkou v případě integrity akrozomu v porovnání s ostatními vzorky.

Signifikantní zlepšení funkčních vlastností spermií (PMot a PMI) oproti vzorku CtrOX bylo pozorováno pouze u vzorku DD. Progresivní motilita je dobrý ukazatelem fertilizačního potenciálu a vykazuje značnou korelaci se schopností spermie proniknout do oocytu (Gadea, 2005). Procento spermií s neporušenou plazmatickou membránou koreluje s fertilizačním potenciálem nejen kančích spermií (Berger et al., 1996; Gil et al., 2008; Sutkeviciene et al., 2009), ale i spermií dalších druhů savců (Brito et al., 2003; Ramu et Jeyendran, 2013). Stejně jako předchozí parametry, na správnou funkci spermií má vliv integrita akrozomu (Stival et al., 2016). I přesto, že vzorek s SNP vykazoval všeobecně vyšší hodnoty kvalitativních parametrů spermií (TMot, PMot a PMI) než CtrOX, rozdíly nebyly signifikantní. Ve studii Hellstrom et al. (1994) byl aplikován SNP při 50 a 100 nM koncentraci na lidské spermie po rozmrazení, přičemž byl pozorován pozitivní účinek na viabilitu a motilitu spermií a zároveň snížená míra lipidové peroxidace. Odlišný účinek SNP při dané koncentraci lze vysvětlit přítomností indukovaného oxidačního stresu v naší studii, který zamaskoval pozitivní efekt SNP. Reakcí NO s ROS vzniká peroxynitrit, který při mikromolání koncentraci snižuje motilitu spermií a zvyšuje míru lipidové peroxidace u kančích spermií (Serrano et al., 2020). Řada studií

zkoumajících účinek NO na spermie prokázala jeho spoluúčast na akrozomální reakci (Aquila et al., 2011; Staicu et Parra, 2017). V případě SNP byla prokázána schopnost spustit akrozomální reakci u lidských (Revelli et al., 2001; Sengoku et al., 1998) a kančích spermií (Funahashi, 2002; Hou et al., 2008). U vzorku SNP byla pozorována zajímavá odlišnost v podobě zvýšené směrodatné odchylky u procenta spermií s neporušeným akrozomem. Nabízí se spekulace, že mohlo dojít k částečné destabilizaci membrány akrozomu u vzorku s 100 nM SNP. V předcházející studii (Pintus et al., 2020), Na_2S vykazoval pozitivní efekt na TMot při nejnižší koncentraci (3 μM), ale při vyšší koncentraci snižoval kvalitativní vlastnosti kančích spermií v přítomnosti oxidačního stresu. Tyto výsledky naznačují, že dávka SNP s positivním účinkem na kančí spermie v in vitro podmínkách se pohybuje v rozmezí nízkých mikromolárních koncentrací.

Výsledky tohoto experimentu naznačují, že účinek obou donorů v DD vzorku se podobá více vzorku s SNP než vzorku s NaHS u kančích spermií vystavených oxidačnímu stresu. To vede ke spekulaci, že ve vzorku DD dochází k formování účinnějšího metabolitu, který napodobuje účinek NO. Nicméně tato hypotéza se musí ověřit.

Uvedené výsledky jsou součástí publikace (viz Přílohy):

Kadlec, M., Pintus, E., Ros-Santaella, J. L. 2022. The Interaction of NO and H2S in Boar Spermatozoa under Oxidative Stress. Animals. 12 (5). 602. doi: 10.3390/ani12050602

6 Závěr

Oxidativní stres je stále jednou z hlavních překážek v širším využití technik asistované reprodukce v chovu prasat. Zároveň je oxidační stres spojován s běžnými poruchami reprodukce (e.g. varikokéla). Modulace oxidačního stresu je proto stále aktuální téma nejen v chovu hospodářských zvířat, ale je i v centru pozornosti asistované reprodukce lidí. V relativně nedávné době se dostaly do centra pozornosti i gasotransmitery, které představují plynné signální molekuly podílející se na řadě buněčných procesů. V této práci byly zkoumány možnosti využití dvou gasotransmiterů, oxidu dusnatého a sulfanu, k modulaci oxidačního stresu u kančích gamet. Oba gasotransmitery mají schopnost vstupovat do redoxních reakcí a byly u nich pozorovány antioxidační vlastnosti za specifických podmínek. Zatímco o úloze oxidu dusnatého v biologii spermií již byla věnována řada studií, mnoho otázek zůstává nezodpovězeno v případě sulfanu.

Selektivní inhibitor iNOS, aminoguanidin, vykazuje antioxidační vlastnosti a lze jej využít k omezení nadměrné produkce NO a akumulace ROS. Za využití aminoguanidinu se podařilo omezit oxidační stres a jeho negativní dopad na funkční vlastnosti spermií v in vitro podmínkách. Dále byly zkoumány antioxidační vlastnosti dvou donorů sulfanu. Anorganická sůl Na_2S, která představuje tradiční donor sulfanu, byla porovnávána se syntetickým donorem sulfanu GYY4137, jež produkuje menší množství sulfanu v delším časovém úsek a lépe reflektuje fyziologickou produkcí sulfanu. Na základě výsledků byl prokázán rozdílný způsob účinku pomalého (Na_2S) a rychlého (GYY4137) donoru sulfanu. Oba donory sulfanu vykazují antioxidační vlastnosti, avšak optimální koncentrace se liší v závislosti na typu donoru.

Aktuální výzkum somatických buněk odhalil úzké vztahy, které mezi těmito dvěma gasotransmitery panují, kdy přítomnost jednoho gasotransmiteru ovlivňuje účinek gasotransmiteru druhého. Dostupné informace v rámci této problematiky vychází převážně z vědeckých prací zkoumajících somatické buňky. V závěru byla ověřena hypotéza, že smíchání donorů NO a H_2S povede k zesílení účinku na kančí spermie vystavené oxidačnímu stresu v porovnání se vzorky, u nichž je aplikován pouze jeden ze dvou donorů. Pouze při simultánní aplikací donorů došlo ke zlepšení funkčních vlastností spermií.

Každá z publikovaných prací přinesla nové poznatky v oblasti vztahů gasotransmiterů, oxidačního stresu a kančích spermií. Aminoguanidin se prokázal jako slibné antioxidační činidlo k potlačení negativního dopadu oxidačního stresu u kančích spermií. Poprvé byly definovány antioxidační vlastnosti dvou donorů sulfanu (Na_2S, H_2S) a zároveň byl prokázán divergentní účinek pomalého a rychlého donoru sulfanu. Poslední dvě publikované studie podtrhují nutnost dalšího výzkumu gasotransmiterů v rámci samčích gamet, a to především z hlediska vzájemné interakce obou gasotransmiterů. Závěrečný výzkum poskytl nový pohled na problematiku gasotransmiterů, spermií

a oxidačního stresu. Další výzkum by mohl navázat analýzou metabolitů vznikajících reakcí donorů NO a H_2S v in vitro podmínkách. Také by bylo vhodné otestovat vliv na spermie u alternativních donorů obou gasotransmiterů v rozdílných koncentracích.

7 Literatura

Abbasi, M., Alizadeh, R., Abolhassani, F., Amidi, F., Hassanzadeh, G., Ejtemaei Mehr, S., Dehpour, A. R. 2011. Aminoguanidine improves epididymal sperm parameters in varicocelized rats. Urologia Internationalis. 86 (3). 302–306. doi: 10.1159/000322154.

Abbasi, M., Alizadeh, R., Abolhassani, F., Amidi, F., Ragerdi, K. I., Fazelipour, S., Hoshino, Y., Sato, E., Dehpour, A. R. 2011. Effect of aminoguanidine in sperm DNA fragmentation in varicocelized rats: Role of nitric oxide. Reproductive Sciences. 18 (6). 545–550. doi: 10.1177/1933719110393028.

Abo-Salem, O. M. 2012. The protective effect of aminoguanidine on doxorubicin-induced nephropathy in rats. Journal of Biochemical and Molecular Toxicology. 26 (1). 1–9. doi: 10.1002/jbt.20422.

Abraham, P., Rabi, S. 2011. Protective effect of aminoguanidine against cyclophosphamide-induced oxidative stress and renal damage in rats. Redox Report. 16 (1). 8–14. doi: 10.1179/174329211X12968219310837.

Agarwal, A., Rana, M., Qiu, E., Al Bunni, H., Bui, A. D., Henkel, R. 2018. Role of oxidative stress, infection and inflammation in male infertility. Andrologia. 50 (11). 1–13. doi: 10.1111/and.13126.

Aitken, R. J., Jones, K. T., Robertson, S. A. 2012. Reactive oxygen species and sperm function-in sickness and in health. Journal of Andrology. 33 (6). 1096–1106. doi: 10.2164/jandrol.112.016535.

Aitken, R J 1995. Free radicals, lipid peroxidation and sperm function. Reproduction, Fertility, and Development. 7 (4). 659–668. doi: 10.1071/RD9950659.

Aitken, R. J. 2017. Reactive oxygen species as mediators of sperm capacitation and pathological damage. Molecular Reproduction and Development. 84 (10). 1039–1052. doi: 10.1002/mrd.22871.

Aitken, R. J., Gibb, Z., Baker, M. A., Drevet, J., Gharagozloo, P. 2016. Causes and consequences of oxidative stress in Spermatozoa. Reproduction, Fertility and Development. 28 (1–2). 1–10. doi: 10.1071/RD15325.

Alizadeh, N., Abbasi, M., Abolhassani, F., Amidi, F., Mahmoudi, R., Hoshino, Y., Sato, E., Ragerdikashani, I. 2010. Effects of aminoguanidine on infertile varicocelized rats: A functional and morphological study. Daru : Journal of Faculty of Pharmacy, Tehran University of Medical Sciences. 18 (1). 51–6.

Alizadeh, R., Navid, S., Abbasi, N., Yari, A., Mazaheri, Z., Daneshi, E., Agarwal, A., Abbasi, M. 2016. The effect of aminoguanidine on sperm motility and mitochondrial membrane potential in varicocelized rats. Iranian Journal of Basic Medical Sciences. 19 (12). 1279–1284. doi: 10.22038/ijbms.2016.7908.

Almog, T., Naor, Z. 2008. Mitogen activated protein kinases (MAPKs) as regulators of spermatogenesis and spermatozoa functions. Molecular and Cellular Endocrinology. 282 (1–2). 39–44. doi: 10.1016/j.mce.2007.11.011.

Am-in, N., Kirkwood, R. N., Techakumphu, M., Tantasuparuk, W. 2011. Lipid profiles of sperm and seminal plasma from boars having normal or low sperm motility. Theriogenology. 75 (5). 897–903.

doi: 10.1016/j.theriogenology.2010.10.032.

Andrews, K. L., Lumsden, N. G., Farry, J., Jefferis, A. M., Kemp-Harper, B. K., Chin-Dusting, J. P. F. 2015. Nitroxyl: A vasodilator of human vessels that is not susceptible to tolerance. Clinical Science. 129 (2). 179–187. doi: 10.1042/CS20140759.

Aquila, S., Giordano, F., Guido, C., Rago, V., Carpino, A. 2011. Nitric oxide involvement in the acrosome reaction triggered by leptin in pig sperm. Reproductive Biology and Endocrinology : RB&E. 9 (1). 133. doi: 10.1186/1477-7827-9-133.

Bansal, A. K., Bilaspuri, G. S. 2011. Impacts of Oxidative Stress and Antioxidants on Semen Functions. Veterinary Medicine International. 2011. 1–7. doi: 10.4061/2011/686137.

Barranco, I., Tvarijonaviciute, A., Perez-patiño, C., Parrilla, I. 2015. High total antioxidant capacity of the porcine seminal plasma (SP-TAC) relates to sperm survival and fertility. Nature Publishing Group. (December). 1–9. doi: 10.1038/srep18538.

Bahmanzadeh, M., Abolhassani, F., Amidi, F., Ejtemaiemehr, S., Salehnia, M., Abbasi, M. 2008. The effects of nitric oxide synthase inhibitor (L-NAME) on epididymal sperm count, motility, and morphology in varicocelized rat. Daru. 16 (1). 23–28. doi: 10.1016/j.niox.2008.06.119.

Benetti, L. R., Campos, D., Gurgueira, S. A., Vercesi, A. E., Guedes, C. E. V., Santos, K. L., Wallace, J. L., Teixeira, S. A., Florenzano, J., Costa, S. K. P., Muscará, M. N., Ferreira, H. H. A. 2013. Hydrogen sulfide inhibits oxidative stress in lungs from allergic mice in vivo. European Journal of Pharmacology. 698 (1–3). 463–469. doi: 10.1016/j.ejphar.2012.11.025.

Berenyiova, A., Grman, M., Mijuskovic, A., Stasko, A., Misak, A., Nagy, P., Ondriasova, E., Cacanyiova, S., Brezova, V., Feelisch, M., Ondrias, K. 2015. The reaction products of sulfide and S-nitrosoglutathione are potent vasorelaxants. Nitric Oxide - Biology and Chemistry. 46. 123–130. doi: 10.1016/j.niox.2014.12.008.

Berger, T., Anderson, D. L., Penedo, M. C. T. 1996. Porcine sperm fertilizing potential in relationship to sperm functional capacities. Animal Reproduction Science. 44 (4). 231–239. doi: 10.1016/0378-4320(96)01565-5.

Bernabò, N., Pistilli, M. G., Mattioli, M., Barboni, B. 2010. Role of TRPV1 channels in boar spermatozoa acquisition of fertilizing ability. Molecular and Cellular Endocrinology. 323 (2). 224–231. doi: 10.1016/j.mce.2010.02.025.

Bianco, C. L., Toscano, J. P., Bartberger, M. D., Fukuto, J. M. 2017. The chemical biology of HNO signaling. Archives of Biochemistry and Biophysics. 617. 129–136. doi: 10.1016/j.abb.2016.08.014.

Björkgren, I., Lishko, P. V. 2017. Fertility and TRP Channels. Neurobiology of TRP Channels. p. 213–228.

Bonet, S., Casas, I., Holt, W. V. 2013. Boar ReproductionBoar Reproduction. Springer Science & Business Media. Berlin. ISBN: 9783642350481.

Brewis, I. A., Gadella, B. M. 2009. Sperm surface proteomics: From protein lists to biological function. Molecular Human Reproduction. 16 (2). 68–79. doi: 10.1093/molehr/gap077.

Brito, L. F. C., Barth, A. D., Bilodeau-Goeseels, S., Panich, P. L., Kastelic, J. P. 2003. Comparison of methods to evaluate the plasmalemma of bovine sperm and their relationship with in vitro fertilization rate. Theriogenology. 60 (8). 1539–1551. doi: 10.1016/S0093-691X(03)00174-2.

Broniowska, K. A., Diers, A. R., Hogg, N. 2013. S-Nitrosoglutathione. Biochimica et Biophysica Acta - General Subjects. 1830 (5). 3173–3181. doi: 10.1016/j.bbagen.2013.02.004.

Bruce King, S. 2013. Potential biological chemistry of hydrogen sulfide (H2S) with the nitrogen oxides. Free Radical Biology and Medicine. 55. 1–7. doi: 10.1016/j.freeradbiomed.2012.11.005.

Burkin, H. R., Miller, D. J. 2000. Zona pellucida protein binding ability of porcine sperm during epididymal maturation and the acrosome reaction. Developmental Biology. 222 (1). 99–109. doi: 10.1006/dbio.2000.9707.

Cai, H., Li, Z., Davis, M. E., Kanner, W., Harrison, D. G., Dudley, S. C. 2003. Akt-dependent phosphorylation of serine 1179 and mitogen-activated protein kinase kinase/extracellular signal-regulated kinase 1/2 cooperatively mediate activation of the endothelial nitric-oxide synthase by hydrogen peroxide. Molecular Pharmacology. 63 (2). 325–331. doi: 10.1124/mol.63.2.325.

Cao, X., Cui, Y., Zhang, X., Lou, J., Zhou, J., Bei, H., Wei, R. 2018. Proteomic profile of human spermatozoa in healthy and asthenozoospermic individuals. Reproductive Biology and Endocrinology. 16 (1). 4–11. doi: 10.1186/s12958-018-0334-1.

Cerolini, S., Maldjian, A., Surai, P., Noble, R. 2000. Viability , susceptibility to peroxidation and fatty acid composition of boar semen during liquid. 99–111.

Cisneros-Mejorado, A., Hernández-Soberanis, L., Islas-Carbajal, M. C., Sánchez, D. 2014. Capacitation and Ca2+ influx in spermatozoa: Role of CNG channels and protein kinase G. Andrology. 2 (1). 145–154. doi: 10.1111/j.2047-2927.2013.00169.x.

Cooper, C. E., Brown, G. C. 2008. The inhibition of mitochondrial cytochrome oxidase by the gases carbon monoxide, nitric oxide, hydrogen cyanide and hydrogen sulfide: Chemical mechanism and physiological significance. Journal of Bioenergetics and Biomembranes. 40 (5). 533–539. doi: 10.1007/s10863-008-9166-6.

Corbett, J. A., Tilton, R. G., Chang, K., Hasan, K. S., Ido, Y., Wang, J. L., Sweetland, M. A., Lancaster, J. R., Williamson, J. R., McDaniel, M. L. 1992. Aminoguanidine, a novel inhibitor of nitric oxide formation, prevents diabetic vascular dysfunction. Diabetes. 41 (4). 552–556. doi: 10.2337/diabetes.41.4.552.

Cortese-Krott, M. M., Fernandez, B. O., Kelm, M., Butler, A. R., Feelisch, M. 2015. On the chemical biology of the nitrite/sulfide interaction. Nitric Oxide - Biology and Chemistry. 46. 14–24. doi: 10.1016/j.niox.2014.12.009.

Cortese-Krott, M. M., Fernandez, B. O., Santos, J. L. T., Mergia, E., Grman, M., Nagy, P., Kelm, M., Butler, A., Feelisch, M. 2014. Nitrosopersulfide (SSNO-) accounts for sustained NO bioactivity of S-nitrosothiols following reaction with sulfide. Redox Biology. 2 (1). 234–244. doi: 10.1016/j.redox.2013.12.031.

Courderot-Masuyer, C., Dalloz, F., Maupoil, V., Rochette, L. 1999. Antioxidant properties of aminoguanidine. Fundamental and Clinical Pharmacology. 13 (5). 535–540. doi: 10.1111/j.1472-

8206.1999.tb00358.x.

Dacheux, J. L., Castella, S., Gatti, J. L., Dacheux, F. 2005. Epididymal cell secretory activities and the role of proteins in boar sperm maturation. Theriogenology. 63 (2 SPEC. ISS.). 319–341. doi: 10.1016/j.theriogenology.2004.09.015.

de Lamirande, E., Gagnon, C. 1995. Impact of reactive oxygen species on spermatozoa: a balancing act between beneficial and detrimental effects. Human Reproduction. 10 (suppl 1). 15–21. doi: 10.1093/humrep/10.suppl_1.15.

de Lamirande, E., O'Flaherty, C. 2008. Sperm activation: Role of reactive oxygen species and kinases. Biochimica et Biophysica Acta - Proteins and Proteomics. 1784 (1). 106–115. doi: 10.1016/j.bbapap.2007.08.024.

Di, T., Sullivan, J. A., Magness, R. R., Zhang, L., Bird, I. M. 2001. Pregnancy-specific enhancement of agonist-stimulated ERK-1/2 signaling in uterine artery endothelial cells increases Ca 2+ sensitivity of endothelial nitric oxide synthase as well as cytosolic phospholipase A 2. Endocrinology. 142 (7). 3014–26. doi: 10.1210/endo.142.7.8278.

Di Villa Bianca, R. D. E., Sorrentino, R., Maffia, P., Mirone, V., Imbimbo, C., Fusco, F., De Palma, R., Ignarro, L. J., Cirino, G. 2009. Hydrogen sulfide as a mediator of human corpus cavernosum smooth-muscle relaxation. Proceedings of the National Academy of Sciences of the United States of America. 106 (11). 4513–4518. doi: 10.1073/pnas.0807974105.

Dixit, V. D., Parvizi, N. 2001. Nitric oxide and the control of reproduction. Animal Reproduction Science. 65 (1–2). 1–16. doi: 10.1016/S0378-4320(00)00224-4.

Eroglu, C., Yildiz, O. G., Saraymen, R., Soyuer, S., Kilic, E., Ozcan, S. 2008. Aminoguanidine ameliorates radiation-induced oxidative lung damage in rats. Clinical & Investigative Medicine. 31 (4). 182. doi: 10.25011/cim.v31i4.4778.

Esfandiari, N., Saleh, R. A., Blaut, A. P., Sharma, R. K., Nelson, D. R., Thomas, A. J., Falcone, T., Agarwal, A. 2002. Effects of temperature on sperm motion characteristics and reactive oxygen species. International Journal of Fertility and Women's Medicine. 47 (5). 227–233.

Evans, R. W., Weaver, D. E., Clegg, E. D. 1980. Diacyl, alkenyl, and alkyl ether phospholipids in ejaculated, in utero-, and in vitro-incubated porcine spermatozoa. Journal of Lipid Research. 21 (2). 223–228.

Filipovic, M. R., Miljkovic, J. L., Nauser, T., Royzen, M., Klos, K., Shubina, T., Koppenol, W. H., Lippard, S. J., Ivanović-Burmazović, I. 2012. Chemical characterization of the smallest S-nitrosothiol, HSNO; Cellular cross-talk of H2S and S-nitrosothiols. Journal of the American Chemical Society. 134 (29). 12016–12027. doi: 10.1021/ja3009693.

Flesch, F. M., Gadella, B. M. 2000. Dynamics of the mammalian sperm plasma membrane in the process of fertilization. Biochemistry and Biophysic. Biochim Biophys Acta. 1469 (3). 197–235.

Förstermann, U., Kleinert, H. 1995. Nitric oxide synthase: expression and expressional control of the three isoforms. Naunyn-Schmiedeberg's Archives of Pharmacology. 352 (4). 351–364. doi: 10.1007/BF00172772.

Fu, M., Zhang, W., Wu, L., Yang, G., Li, H., Wang, R. 2012. Hydrogen sulfide (H_2S) metabolism in mitochondria and its regulatory role in energy production. Proceedings of the National Academy of Sciences of the United States of America. 109 (8). 2943–2948. doi: 10.1073/pnas.1115634109.

Funahashi, H. 2002. Induction of capacitation and the acrosome reaction of boar spermatozoa by L-arginine and nitric oxide synthesis associated with the anion transport system. Reproduction. 124 (6). 857–864. doi: 10.1530/rep.0.1240857.

Funahashi, H., Sano, T. 2005. Select antioxidants improve the function of extended boar semen stored at 10°C. Theriogenology. 63 (6). 1605–1616. doi: 10.1016/j.theriogenology.2004.06.016.

Funahashi, H. 2003. Polyspermic penetration in porcine IVM-IVF systems. Reproduction, Fertility and Development. 15 (3–4). 167–177. doi: 10.1071/RD02076.

Gadea, J. 2003. Review: semen extenders used in the artificial inseminarion of swine. Spanish Journal of Agricultural Research. 1 (2). 17. doi: 10.5424/sjar/2003012-17.

Gadea, J. 2005. Sperm factors related to in vitro and in vivo porcine fertility. Theriogenology. 63 (2). 431–44. doi: 10.1016/j.theriogenology.2004.09.023.

Gadella, B. M. 2017. Reproductive tract modifications of the boar sperm surface. Molecular Reproduction and Development. 84 (9). 822–831. doi: 10.1002/mrd.22821.

Gangwar, D. K., Atreja, S. K. 2015. Signalling Events and Associated Pathways Related to the Mammalian Sperm Capacitation. Reproduction in Domestic Animals. 50 (5). 705–711. doi: 10.1111/rda.12541.

Gao, D. D., Xu, J. W., Qin, W. B., Peng, L., Qiu, Z. E., Wang, L. L., Lan, C. F., Cao, X. N., Xu, J. B., Zhu, Y. X., Tang, Y. G., Zhang, Y. L., Zhou, W. L. 2019. Cellular mechanism underlying hydrogen sulfide mediated epithelial K+ secretion in rat epididymis. Frontiers in Physiology. 9. 1886. doi: 10.3389/fphys.2018.01886.

Garcia-Gil, N., Pinart, E., Sancho, S., Badia, E., Bassols, J., Kádár, E., Briz, M., Bonet, S. 2002. The cycle of the seminiferous epithelium in Landrace boars. Animal Reproduction Science. 73 (3–4). 211–225. doi: 10.3389/fphys.2018.01886.

García-Ortiz, A., Serrador, J. M. 2018. Nitric Oxide Signaling in T Cell-Mediated Immunity. Trends in Molecular Medicine. 24 (4). 412–427. doi: 10.1016/j.molmed.2018.02.002.

Gil, M. A., Almiñana, C., Roca, J., Vázquez, J. M., Martínez, E. A. 2008. Boar semen variability and its effects on IVF efficiency. Theriogenology. 70 (8). 1260–1268. doi: 10.1016/j.theriogenology.2008.06.004.

Giles, G. I., Nasim, M. J., Ali, W., Jacob, C. 2017. The reactive sulfur species concept: 15 years on. Antioxidants. 6 (2). 1–29. doi: 10.3390/antiox6020038.

Gomez, E., Buckingham, D. W., Brindle, J., Lanzafame, F., Irvine, D. S., Aitken, R. J. 2021. Development of an image analysis system to monitor the retention of residual cytoplasm by human spermatozoa: correlation with biochemical markers of the cytoplasmic space, oxidative stress, and sperm function. Journal of Andrology. 17 (3). 276–87.

Gupta, R. K., Swain, D. K., Singh, V., Anand, M., Choudhury, S., Yadav, S., Saxena, A., Garg, S. K. 2018. Molecular characterization of voltage-gated potassium channel (Kv) and its importance in functional dynamics in bull spermatozoa. Theriogenology. 114. 229–236. doi: 10.1016/j.theriogenology.2018.03.030.

Hellstrom, W. J. G., Bell, M., Wang, R., Sikka, S. C. 1994. Effect of sodium nitroprusside on sperm motility, viability, and lipid peroxidation. Fertility and Sterility. 61 (6). 1117–1122. doi: 10.1016/S0015-0282(16)56766-1.

Herrero, M. B., Goin, J. C., Boquet, M., Canteros, M. G., Franchi, A. M., Perez Martinez, S., Polak, J. M., Viggiano, J. M., Gimeno, M. A. F. 1997. The nitric oxide synthase of mouse spermatozoa. FEBS Letters. 411 (1). 39–42. doi: 10.1016/S0014-5793(97)00570-X.

Herrero, B. M., Chatterjee, S., Lefièvre, L., De Lamirande, E., Gagnon, C. 2000. Nitric oxide interacts with the cAMP pathway to modulate capacitation of human spermatozoa. Free Radical Biology and Medicine. 29 (6). 522–536. doi: 10.1016/S0891-5849(00)00339-7Herrero, M. B., Gagnon, C. 2001. Nitric oxide: a novel mediator of sperm function. Journal of Andrology. 22 (3). 349–56. doi: 10.1002/j.1939-4640.2001.tb02188.x.

Herrero, M., Perez, M. S., Viggiano, J., Polak, J., De, G. M. 1996. Localization by indirect immunofluorescence of nitric oxide synthase in mouse and human spermatozoa. Reproduction, Fertility and Development. 8 (5). 931. doi: 10.1071/RD9960931.

Hou, M. L., Huang, S. Y., Lai, Y. K., Lee, W. C. 2008. Geldanamycin augments nitric oxide production and promotes capacitation in boar spermatozoa. Animal Reproduction Science. 104 (1). 56–68. doi: 10.1016/j.anireprosci.2007.01.006.

Ivanovic-Burmazovic, I., Filipovic, M. R. 2019. Saying NO to H_2S: A Story of HNO, HSNO, and $SSNO^-$. Inorganic Chemistry. 58 (7). 4039–4051. doi: 10.1021/acs.inorgchem.8b02592. editorial.

Janakiram, N. B., Rao, C. V 2012. iNOS-selective inhibitors for cancer prevention: promise and progress. Future Medicinal Chemistry. 4 (17). 2193–2204. doi: 10.4155/fmc.12.168.

Jia, Y., Castellanos, J., Wang, C., Sinha-Hikim, I., Lue, Y., Swerdloff, R. S., Sinha-Hikim, A. P. 2009. Mitogen-Activated Protein Kinase Signaling in Male Germ Cell Apoptosis in the Rat1. Biology of Reproduction. 80 (4). 771–780. doi: 10.1095/biolreprod.108.072843.

Johnson, L. A., Weitze, K. F., Fiser, P., Maxwell, W. M. C. 2006. Storage of boar semen Animal reproduction science. 62 (1-3). 143-172.

Jung, M., Rüdiger, K., Schulze, M. 2015. In Vitro Measures for Assessing Boar Semen Fertility. Reproduction in Domestic Animals. 50. 20–24. doi: 10.1111/rda.12533.

Kastelic, J. P., Thundathil, J. C. 2008. Breeding Soundness Evaluation and Semen Analysis for Predicting Bull Fertility. Reproduction in Domestic Animals. 43 (SUPPL.2). 368–373. doi: 10.1111/j.1439-0531.2008.01186.x.

Kessopoulou, E., Tomlinson, M. J., Barratt, C. L. R., Bolton, A. E., Cooke, I. D. 1992. Origin of reactive oxygen species in human semen: spermatozoa or leucocytes? Reproduction. 94 (2). 463–470. doi: 10.1530/jrf.0.0940463.

Kimura, H. 2014. Hydrogen sulfide and polysulfides as biological mediators. Molecules. 19 (10). 16146–16157. doi: 10.3390/molecules191016146.

Kimura, Y., Goto, Y.-I., Kimura, H. 2010. Hydrogen Sulfide Increases Glutathione Production and Suppresses Oxidative Stress in Mitochondria. Antioxidants & Redox Signaling. 12 (1). 1–13. doi: 10.1089/ars.2008.2282.

Kolluru, G. K., Shen, X., Bir, S. C., Kevil, C. G. 2013. Hydrogen sulfide chemical biology: Pathophysiological roles and detection. Nitric Oxide. 35. 5–20. doi: 10.1016/j.niox.2013.07.002.

Kolluru, G. K., Shen, X., Yuan, S., Kevil, C. G. 2017. Gasotransmitter heterocellular signaling. Antioxidants and Redox Signaling. 26 (16). 936–960. doi: 10.1089/ars.2016.6909.

Kolluru, G. K., Yuan, S., Shen, X., Kevil, C. G. 2015. H_2S regulation of nitric oxide metabolism. Methods in Enzymology. 554. 271–297.

Kowalowka, M., Wysocki, P., Fraser, L., Strzezek, J. 2008. Extracellular Superoxide Dismutase of Boar Seminal Plasma. 496. 490–496. doi: 10.1111/j.1439-0531.2007.00943.x.

Kumar, A., Mishra, A. K., Swain, D. K., Singh, V., Yadav, S., Saxena, A. 2018. Role of transient receptor potential channels in regulating spermatozoa functions: A mini-review. Veterinary World. 11 (11). 1618–1623. doi: 10.14202/vetworld.2018.1618-1623.

Leahy, T., Gadella, B. M. 2011. Sperm surface changes and physiological consequences induced by sperm handling and storage. Reproduction. 142 (6). 759–778. doi: 10.1530/REP-11-0310.

Lee, N. P. Y., Cheng, C. Y. 2004. Nitric Oxide/Nitric Oxide Synthase, Spermatogenesis, and Tight Junction Dynamics1. Biology of Reproduction. 70 (2). 267–276. doi: 10.1095/biolreprod.103.021329.

Lefièvre, L., Chen, Y., Conner, S. J., Scott, J. L., Publicover, S. J., Ford, W. C. L., Barratt, C. L. R. 2007. Human spermatozoa contain multiple targets for protein S-nitrosylation: An alternative mechanism of the modulation of sperm function by nitric oxide? Proteomics. 7 (17). 3066–3084. doi: 10.1002/pmic.200700254.

Li, G., Xie, Z. Z., Chua, J. M. W., Wong, P. C., Bian, J. 2015. Hydrogen sulfide protects testicular germ cells against heat-induced injury. Nitric Oxide - Biology and Chemistry. 46. 165–171. doi: 10.1016/j.niox.2014.10.005.

Li, L., Rose, P., Moore, P. K. 2011. Hydrogen Sulfide and Cell Signaling. Annual Review of Pharmacology and Toxicology. 51 (1). 169–187. doi: 10.1146/annurev-pharmtox-010510-100505.

Liman, N., Alan, E. 2016. Region-specific localization of NOS isoforms and NADPH-diaphorase activity in the intratesticular and excurrent duct systems of adult domestic cats (Felis catus). Microscopy Research and Technique. 79 (3). 192–208. doi: 10.1002/jemt.22619.

Lind, M., Hayes, A., Caprnda, M., Petrovic, D., Rodrigo, L., Kruzliak, P., Zulli, A. 2017. Inducible nitric oxide synthase: Good or bad? Biomedicine and Pharmacotherapy. 93. 370–375. doi: 10.1016/j.biopha.2017.06.036.

Ljiljak, D., Tramiak, T., Smiljan, N., Kuna, K., Radoji, A. 2012. Sperm Cell in ART. In: Wu, B. 2012.

Advances in embryotransfer. Intech. Croatia. 65-72. ISBN: 9789535103189.

Lopez, R. 2015. An Approach to the Factors Related to Sperm Capacitation Process. Andrology-Open Access. 4 (1). 1721–1736. doi: 10.4172/2167-0250.1000128.

Love, C. C. 2016. Modern Techniques for Semen Evaluation. Veterinary Clinics of North America: Equine Practice. 32 (3). 531–546. doi: 10.1016/j.cveq.2016.07.006.

Lundberg, J. O., Gladwin, M. T., Weitzberg, E. 2015. Strategies to increase nitric oxide signalling in cardiovascular disease. Nature Reviews Drug Discovery. 14 (9). 623–641. doi: 10.1038/nrd4623.

Mann, T., Lutwak-Mann, C. 1981. Male Reproductive Function and Semen. London. Springer London. ISBN: 978-1-4471-1302-7.

Matoušková, M., Ruttkay-Nedecký, B., Kizek, R. 2014. Antioxidační enzymy - biochemické markery oxidačního stresu. Journal of Metallomics and Nanotechnologies. 3. 53–56.

Miki, K., Clapham, D. E. 2013. Rheotaxis guides mammalian sperm. Current Biology. 23 (6). 443–452. doi: 10.1016/j.cub.2013.02.007.

Miki, K., Qu, W., Goulding, E. H., Willis, W. D., Bunch, D. O., Strader, L. F., Perreault, S. D., Eddy, E. M., O'Brien, D. A. 2004. Glyceraldehyde 3-phosphate dehydrogenase-S, a sperm-specific glycolytic enzyme, is required for sperm motility and male fertility. Proceedings of the National Academy of Sciences of the United States of America. 101 (47). 16501–16506. doi: 10.1073/pnas.0407708101.

Miraglia, E., Rullo, M. L., Bosia, A., Massobrio, M., Revelli, A., Ghigo, D. 2007. Stimulation of the nitric oxide/cyclic guanosine monophosphate signaling pathway elicits human sperm chemotaxis in vitro. Fertility and Sterility. 87 (5). 1059–1063. doi: 10.1016/j.fertnstert.2006.07.1540.

Mishanina, T. V, Libiad, M., Banerjee, R. 2015. Biogenesis of reactive sulfur species for signaling by hydrogen sulfide oxidation pathways. Nature Chemical Biology. 11 (7). 457–464. doi: 10.1038/nchembio.1834.

Misko, T. P., Moore, W. M., Kasten, T. P., Nickols, G. A., Corbett, J. A., Tilton, R. G., McDaniel, M. L., Williamson, J. R., Currie, M. G. 1993. Selective inhibition of the inducible nitric oxide synthase by aminoguanidine. European Journal of Pharmacology. 233 (1). 119–125. doi: 10.1016/0014-2999(93)90357-N.

Molina, L. C. P., Luque, G. M., Balestrini, P. A., Marín-Briggiler, C. I., Romarowski, A., Buffone, M. G. 2018. Molecular basis of human sperm capacitation. Frontiers in Cell and Developmental Biology. 6. 1–23. doi: 10.3389/fcell.2018.00072.

Mundt, N., Spehr, M., Lishko, P. V. 2018. TRPV4 is the temperature-sensitive ion channel of human sperm. ELife. 7. 1–20. doi: 10.7554/eLife.35853.

Musset, B., Clark, R. A., DeCoursey, T. E., Petheo, G. L., Geiszt, M., Chen, Y., Cornell, J. E., Eddy, C. A., Brzyski, R. G., El Jamali, A. 2012. NOX5 in human spermatozoa: Expression, function, and regulation. Journal of Biological Chemistry. 287 (12). 9376–9388. doi: 10.1074/jbc.M111.314955.

Nagpure, B. V., Bian, J. S. 2016. Interaction of Hydrogen Sulfide with Nitric Oxide in the Cardiovascular System. Oxidative Medicine and Cellular Longevity. 2016. doi: 10.1155/2016/6904327.

Nagy, Z. P. 2000. Sperm centriole disfunction and sperm immotility. Molecular and Cellular Endocrinology. 166 (1). 59–62. doi: 10.1016/S0303-7207(00)00298-7.

Nikolopoulou, M., Soucek, D. A., Vary, J. C. 1985. Changes in the lipid content of boar sperm plasma membranes during epididymal maturation. Biochimica et Biophysica Acta. 815 (3). 486–498.

Ning, J. Z., Li, W., Cheng, F., Rao, T., Yu, W. M., Ruan, Y., Yuan, R., Zhang, X. Bin, Du, Y., Xiao, C. C. 2018. The protective effects of GYY4137 on ipsilateral testicular injury in experimentally varicocele-induced rats. Experimental and Therapeutic Medicine. 15 (1). 433–439. doi: 10.3892/etm.2017.5417.

O'Donnell, L., Nicholls, P. K., O'Bryan, M. K., McLachlan, R. I., Stanton, P. G. 2011. Spermiation. Spermatogenesis. 1 (1). 14–35. doi: 10.4161/spmg.1.1.14525.

O'Flaherty, Cristian 2014. Peroxiredoxins: Hidden players in the antioxidant defence of human spermatozoa. Basic and Clinical Andrology. 24 (1). 1–10. doi: 10.1186/2051-4190-24-4.

O'Flaherty, Cristián 2014. The Enzymatic Antioxidant System of Human Spermatozoa. Advances in Andrology. 2014. 1–15. doi: 10.1155/2014/626374.

Oguz, F., Ciftci, O., Aydin, M., Timurkaan, N., Beytur, A., Altintas, R., Parlakpinar, H. 2013. Aminoguanidine prevents testicular damage-induced-2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) in male rats. Andrologia. 45 (4). 225–231. doi: 10.1111/j.1439-0272.2012.01334.x.

Olson, K. R. 2018. H_2S and polysulfide metabolism: Conventional and unconventional pathways. Biochemical Pharmacology. 149 (2). 77–90. doi: 10.1016/j.bcp.2017.12.010.

Olson, K. R. 2019. Hydrogen sulfide, reactive sulfur species and coping with reactive oxygen species. Free Radical Biology and Medicine. 140. 74–83. doi: 10.1016/j.freeradbiomed.2019.01.020.

Olson, K. R., Gao, Y., DeLeon, E. R., Arif, M., Arif, F., Arora, N., Straub, K. D. 2017. Catalase as a sulfide-sulfur oxido-reductase: An ancient (and modern?) regulator of reactive sulfur species (RSS). Redox Biology. 12. 325–339. doi: 10.1016/j.redox.2017.02.021.

Olson, K. R., Straub, K. D. 2016. The role of hydrogen sulfide in evolution and the evolution of hydrogen sulfide in metabolism and signaling. Physiology. 31 (1). 60–72. doi: 10.1152/physiol.00024.2015.

Ondrias, K., Stasko, A., Cacanyiova, S., Sulova, Z., Krizanova, O., Kristek, F., Malekova, L., Knezl, V., Breier, A. 2008. H_2S and HS^- donor NaHS releases nitric oxide from nitrosothiols, metal nitrosyl complex, brain homogenate and murine L1210 leukaemia cells. European Journal of Physiology. 457 (2). 271–279. doi: 10.1007/s00424-008-0519-0.

Otasevic, V., Stancic, A., Korac, A., Jankovic, A., Korac, B. 2020. Reactive oxygen, nitrogen, and sulfur species in human male fertility. A crossroad of cellular signaling and pathology. BioFactors. 46 (2). 206–219. doi: 10.1002/biof.1535.

Peña, F. J., Johannisson, A., Wallgren, M., Rodriguez Martinez, H. 2003. Antioxidant supplementation in vitro improves boar sperm motility and mitochondrial membrane potential after cryopreservation of different fractions of the ejaculate. Animal Reproduction Science. 78 (1–2). 85–98. doi: 10.1016/S0378-4320(03)00049-6.

Philis-Tsimikas, A., Parthasarathy, S., Picard, S., Palinski, W., Witztum, J. L. 1995. Aminoguanidine Has Both Pro-oxidant and Antioxidant Activity Toward LDL. Arteriosclerosis, Thrombosis, and Vascular Biology. 15 (3). 367–376. doi: 10.1161/01.ATV.15.3.367.

Pinart, E., Sancho, S., Briz, M., Bonet, S. 1999. Morphologic study of the testes from spontaneous unilateral and bilateral abdominal cryptorchid boars. Journal of Morphology. 239 (3). 225–243. doi: 10.1002/(SICI)1097-4687(199903)239:3<225::AID-JMOR1>3.0.CO;2-V.

Pinart, E., Sancho, S., Briz, M. D., Bonet, S., Garcia, N., Badia, E. 2000. Ultrastructural study of the boar seminiferous epithelium: Changes in cryptorchidism. Journal of Morphology. 244 (3). 190–202. doi: 10.1002/(SICI)1097-4687(200006)244:3<190::AID-JMOR4>3.0.CO;2-B.

Pintus, E., Jovičić, M., Kadlec, M., Ros-Santaella, J. L. 2020. Divergent effect of fast- and slow-releasing H2S donors on boar spermatozoa under oxidative stress. Scientific Reports. 10 (1). 1–12. doi: 10.1038/s41598-020-63489-4.

Porter, A. G., Jänicke, R. U. 1999. Emerging roles of caspase-3 in apoptosis. Cell Death and Differentiation. 6 (2). 99–104. doi: 10.1038/sj.cdd.4400476.

Radi, R. 2018. Oxygen radicals, nitric oxide, and peroxynitrite: Redox pathways in molecular medicine. Proceedings of the National Academy of Sciences of the United States of America. 115 (23). 5839–5848. doi: 10.1073/pnas.1804932115.

Ramu, S., Jeyendran, R. S. 2013. The hypo-osmotic swelling test for evaluation of sperm membrane integrity. Methods in Molecular Biology. 927. 21–25. doi: 10.1007/978-1-62703-38-0_3.

Revelli, A., Costamagna, C., Moffa, F., Aldieri, E., Ochetti, S., Bosia, A., Massobrio, M., Lindblom, B., Ghigo, D. 2001. Signaling pathway of nitric oxide-induced acrosome reaction in human spermatozoa. Biology of Reproduction. 64 (6). 1708–1712. doi: 10.1095/biolreprod64.6.1708.

Rodriguez, A. L., Soom, A. Van, Arsenakis, I., Maes, D. 2017. Boar management and semen handling factors affect the quality of boar extended semen. Porcine Health Management. 3. 1–12. doi: 10.1186/s40813-017-0062-5.

Rose, P., Dymock, B. W., Moore, P. K. 2015. GYY4137, a Novel Water-Soluble, H_2S-Releasing Molecule. In: Cadenas, E., Packer, L. 2015. Hydrogen Sulfide in Redox Biology. Elsevier. 554. 143–167. ISBN: 9780128015124.

Rose, P., Moore, P. K., Zhu, Y. Z. 2017. H_2S biosynthesis and catabolism: new insights from molecular studies. Cellular and Molecular Life Sciences. 74 (8). 1391–1412. doi: 10.1007/s00018-016-2406-8.

Sancho, S., Vilagran, I. 2013. The Boar Ejaculate : Sperm Function and Seminal Plasma Analyses. In: Bonet, S., Casas, I., Holt, W. V., Yeste, M. 2013. Boar reproduction: fundamentals and new biotechnological trends. Springer. Berlin. Heidelberg. 471–516. ISBN: 9783642350498.

Schieke, S. M., Briviba, K., Klotz, L. O., Sies, H. 1999. Activation pattern of mitogen-activated protein kinases elicited by peroxynitrite: Attenuation by selenite supplementation. FEBS Letters. 448 (2–3). 301–303. doi: 10.1016/S0014-5793(99)00372-5.

Sengoku, K., Tamate, K., Yoshida, T., Takaoka, Y., Miyamoto, T., Ishikawa, M. 1998. Effects of low concentrations of nitric oxide on the zona pellucida binding ability of human spermatozoa. Fertility and Sterility. 69 (3). 522–527. doi: 10.1016/s0015-0282(97)00537-2.

Serrano, R., Garrido, N., Céspedes, J. A., González-Fernández, L., García-Marín, L. J., Bragado, M. J. 2020. Molecular Mechanisms Involved in the Impairment of Boar Sperm Motility by Peroxynitrite-Induced Nitrosative Stress. International Journal of Molecular Sciences. 21 (4). 12–14. doi: 10.3390/ijms21041208.

Shefa, U., Kim, M. S., Jeong, N. Y., Jung, J. 2018. Antioxidant and Cell-Signaling Functions of Hydrogen Sulfide in the Central Nervous System. Oxidative Medicine and Cellular Longevity. 2018. doi: 10.1155/2018/1873962.

Sikka, S. C. 2004. Role of Oxidative Stress and Antioxidants in Andrology and Assisted Reproductive Technology. Journal of Andrology. 25 (1). 5–18. doi: 10.1002/j.1939-4640.2004.tb02751.x.

Silva, J. V., Freitas, M. J., Correia, B. R., Korrodi-Gregório, L., Patrício, A., Pelech, S., Fardilha, M. 2015. Profiling signaling proteins in human spermatozoa: Biomarker identification for sperm quality evaluation. Fertility and Sterility. 104 (4). 845-856.e8. doi: 10.1016/j.fertnstert.2015.06.039.

Singh, A. P., Rajender, S. 2015. CatSper channel, sperm function and male fertility. Reproductive BioMedicine Online. 30 (1). 28–38.

Smulik-Izydorczyk, R., Dębowska, K., Pięta, J., Michalski, R., Marcinek, A., Sikora, A. 2018. Fluorescent probes for the detection of nitroxyl (HNO). Free Radical Biology and Medicine. 128. 69–83. doi: 10.1016/j.freeradbiomed.2018.04.564.

Staicu, F.D., Lopez-Úbeda, R., Romero-Aguirregomezcorta, J., Martínez-Soto, J. C., Matás Parra, C. 2019. Regulation of boar sperm functionality by the nitric oxide synthase/nitric oxide system. Journal of Assisted Reproduction and Genetics. 36 (8). 1721–1736. doi: 10.1007/s10815-019-01526-6.

Staicu, F. D., Parra, C. M. 2017. Nitric Oxide: Key Features in Spermatozoa. In: Saravi, S.S.S. 2017. Nitric Oxide Synthase - Simple Enzyme-Complex Roles. IntechOpen. London. 220. 138–154. ISBN 978-953-51-3164-9.

Stival, C., Puga Molina, L. del C., Paudel, B., Buffone, M. G., Visconti, P. E., Krapf, D. 2016. Sperm Capacitation and Acrosome Reaction in Mammalian Sperm. In: M. G. Buffone. 2016. Sperm Acrosome Biogenesis and Function During Fertilization. 220. 93–106. Springer International Publishing. ISBN: 978-3-319-30565-3.

Storey, B. T. 2008. Mammalian sperm metabolism: oxygen and sugar, friend and foe. The International Journal of Developmental Biology. 52 (5–6). 427–437. doi: 10.1387/ijdb.072522bs.

Suarez, S. S., Ho, H. C. 2003. Hyperactivated motility in sperm. Reproduction in Domestic Animals. 38 (2). 119–124. doi: 10.1046/j.1439-0531.2003.00397.x.

Sun, M., Zhao, Y., Gu, Y., Xu, C. 2010. Neuroprotective actions of aminoguanidine involve reduced

the activation of calpain and caspase-3 in a rat model of stroke. Neurochemistry International. 56 (4). 634–641. doi: 10.1016/j.neuint.2010.01.009.

Sutkeviciene, N., Riskeviciene, V., Januskauskas, A., Zilinskas, H., Andersson, M. 2009. Assessment of sperm quality traits in relation to fertility in boar semen. Acta Veterinaria Scandinavica. 51. 53. doi: 10.1186/1751-0147-51-53.

Szabo, C., Ransy, C., Módis, K., Andriamihaja, M., Murghes, B., Coletta, C., Olah, G., Yanagi, K., Bouillaud, F. 2014. Regulation of mitochondrial bioenergetic function by hydrogen sulfide. Part I. Biochemical and physiological mechanisms. British Journal of Pharmacology. 171 (8). 2099–2122. doi: 10.1111/bph.12369.

Toor, J. S., Sikka, S. C. 2019. Human Spermatozoa and Interactions With Oxidative Stress. In: Henkel, R., Samanta, L., Agarwal, A. 2019. Oxidants, Antioxidants and Impact of the Oxidative Status in Male Reproduction. Elsevier Inc. 45–53. ISBN: 9780128125014.

Türkyilmaz, Z., Gülen, Ş., Sönmez, K., Karabulut, R., Dinçer, S., Başaklar, A. C., Kale, N. 2004. Increased nitric oxide is accompanied by lipid oxidation in adolescent varicocele. International Journal of Andrology. 27 (3). 183–187. doi: 10.1111/j.1365-2605.2004.00474.x.

Vicente-Carrillo, A., Álvarez-Rodríguez, M., Rodríguez-Martínez, H. 2017. The CatSper channel modulates boar sperm motility during capacitation. Reproductive Biology. 17 (1). 69–78. doi: 10.1016/j.repbio.2017.01.001.

Wang, J., Wang, W., Li, S., Han, Y., Zhang, P., Meng, G., Xiao, Y., Xie, L., Wang, X., Sha, J., Chen, Q., Moore, P. K., Wang, R., Xiang, W., Ji, Y. 2018. Hydrogen Sulfide As a Potential Target in Preventing Spermatogenic Failure and Testicular Dysfunction. Antioxidants & Redox Signaling. 28 (16). 1447–1462. doi: 10.1089/ars.2016.6968.

Wang, R. 2010. Hydrogen sulfide: The third gasotransmitter in biology and medicine. Antioxidants and Redox Signaling. 12 (9). 1061–1064. doi: 10.1089/ars.2009.2938.

Wedmann, R., Ivanovic-Burmazovic, I., Filipovic, M. R. 2017. Nitrosopersulfide ($SSNO^-$) decomposes in the presence of sulfide, cyanide or glutathione to give $HSNO/SNO^-$: Consequences for the assumed role in cell signalling. Interface Focus. 7 (2). doi: 10.1098/rsfs.2016.0139.

Weidinger, A., Kozlov, A. V. 2015. Biological activities of reactive oxygen and nitrogen species: Oxidative stress versus signal transduction. Biomolecules. 5 (2). 472–484. doi: 10.3390/biom5020472.

Whiteman, M., Li, L., Rose, P., Tan, C. H., Parkinson, D. B., Moore, P. K. 2010. The Effect of Hydrogen Sulfide Donors on Lipopolysaccharide-Induced Formation of Inflammatory Mediators in Macrophages. Antioxidants & Redox Signaling. 12 (10). 1147–1154. doi: 10.1089/ars.2009.2899.

Wiliński, B., Wiliński, J., Gajda, M., Jasek, E., Somogyi, E., Głowacki, M., Śliwa, L. 2015. Sodium Hydrosulfide Exerts a Transitional Attenuating Effect on Spermatozoa Migration in Vitro. Folia Biologica. 63 (2). 145–9. doi: 10.3409/fb63_2.145.

Wolff, D. J., Lubeskie, A. 1995. Aminoguanidine Is an Isoform-Selective, Mechanism-Based Inactivator of Nitric Oxide Synthase. Archives of Biochemistry and Biophysics. 316 (1). 290–301. doi: 10.1006/abbi.1995.1040.

Xie, Z. Z., Liu, Y., Bian, J. S. 2016. Hydrogen Sulfide and Cellular Redox Homeostasis. Oxidative Medicine and Cellular Longevity. 2016. doi: 10.1155/2016/6043038.

Yildiz, G., Demiryürek, A. T., Sahin-Erdemli, I., Kanzik, I. 1998. Comparison of antioxidant activities of aminoguanidine, methylguanidine and guanidine by luminol-enhanced chemiluminescence. British Journal of Pharmacology. 124 (5). 905–910. doi: 10.1038/sj.bjp.0701924.

Yonezawa, D., Sekiguchi, F., Miyamoto, M., Taniguchi, E., Honjo, M., Masuko, T., Nishikawa, H., Kawabata, A. 2007. A protective role of hydrogen sulfide against oxidative stress in rat gastric mucosal epithelium. Toxicology. 241 (1–2). 11–18. doi: 10.1016/j.tox.2007.07.020.

Yoshida, T., Inoue, R., Morii, T., Takahashi, N., Yamamoto, S., Hara, Y., Tominaga, M., Shimizu, S., Sato, Y., Mori, Y. 2006. Nitric oxide activates TRP channels by cysteine S-nitrosylation. Nature Chemical Biology. 2 (11). 596–607. doi: 10.1038/nchembio821.

Zakošek Pipan, M., Mrkun, J., Kosec, M., Nemec Svete, A., Zrimšek, P. 2014. Superoxide dismutase: A predicting factor for boar semen characteristics for short-term preservation. BioMed Research International. 2014. doi: 10.1155/2014/105280.

Zhao, Y., Zhang, W. D., Liu, X. Q., Zhang, P. F., Hao, Y. N., Li, L., Chen, L., Shen, W., Tang, X. F., Min, L. J., Meng, Q. S., Wang, S. K., Yi, B., Zhang, H. F. 2016. Hydrogen sulfide and/or ammonia reduces spermatozoa motility through AMPK/AKT related pathways. Scientific Reports. 6. 1–11. doi: 10.1038/srep37884.

8 Seznam zkratek

- 3-MST 3-merkaptopyruvat sulfutransferaza
- AG aminoguanidin
- ALH amplituda laterárního vybočení hlavičky spermie
- ATP adenosintrifosfát
- BCF frekvence křížení
- BSA hovězí sérový albumin
- BTS extender Beltsville Thawing Solution
- cAMP cyklický adenosinmonofosfát
- CASA počítačem řízená anlýza spermií
- CAT cystein aminotransferáza
- CBS cystationin β-syntaza
- CFDA karboxyfluorescein diacetát
- cGMP cyklický guanylát monofosfát
- CNG cyklickým nukleotidem aktivované iontové kanály
- CSE cystationin γ-lyaza
- DAO oxidáza D-aminokyselin
- DMSO dimetylsulfoxid
- eNOS endoteliální NOS
- ERK extracelulárně regulované kinázy
- ETHE1 etylmalon-encefalopatický protein 1
- FITC fluorescein-5-isothiokyanát
- G6PDH glukózo-6-fosfát dehydrogenáz
- GAPDH glyceraldehyd 3-fosfát dehydrogenáza
- GPx glutation peroxidáza
- GSNO S-nitrosoglutathion
- GZLM generalizované lineární modely
- HOS test test hypoosmotické bobtnavosti
- iNOS inducibilní NOS
- JNK C-Jun N-terminální kináza
- LIN linearita
- LPS lipopolysacharidy

- MAPK mitogenem aktivované proteinkinázy
- MDA 1,1,3,3-tetrametoxypropan
- MEK mitogenem aktivovaná proteinkináza kináza
- NADPH nikotinamidadenindinukleotid fosfát
- NAR test test integrity apikálního hřebene akrozomu
- nNOS neuronální NOS
- NOS syntáza oxidu dusnatého
- NOX NADPH oxidáza
- PAK protein kináza A
- PBS solný roztok pufrovaný fosfátem
- PDE3 fosfodiesteráza typu 3
- PI3K fosfatidylinositol-3-kináza
- PKG cGMP-dependentní protein kináza
- PNA aglutinin podzemnice olejné
- PUFA polynenasycené mastné kyseliny
- QM kvalita pohybu
- RNS reaktivní formy dusíku
- RNS reaktivní formy dusíku
- ROS reaktivní formy kyslíku
- ROS reaktivní formy kyslíku
- RS reaktivní sloučeniny
- RS reaktivní sloučeniny
- RSS reaktivní formy síry
- RSS reaktivní formy síry
- SAPKs stresem aktivované protein kinázy
- sGC solubilní guanylát cykláza
- sGC solubilní adenylyl cykláza
- SMI index motility spermií
- SMI index motility spermií
- SNP nitroprusid sodný
- SOD superoxid dismutáza
- SQR sulfid chinon oxidoreduktáza
- STR přímost

- TAC celková antioxidační kapacita
- TBARS kyselina thiobarbiturová
- Trolox 6-hydroxy-2,5,7,8-tetrametylchroman-2-karboxylová kyselina
- TRP transient receptor potential iontové kanály
- TRPV TRP vanilloid iontové kanály
- TST thiosulfát transferáza
- VAP rychlost po průměrné trajektorii
- VCL rychlost křivočarého pohybu
- VSL rychlost přímého pohybu

9 Přílohy

Kadlec, M., Pintus, E., Ros-Santaella, J. L. 2022. The Interaction of NO and H_2S in Boar Spermatozoa under Oxidative Stress. Animals. 12 (5). 602. doi: 10.3390/ani12050602.

Kadlec, M., Ros-Santaella, J. L., Pintus, E. 2020. The Roles of NO and H_2S in Sperm Biology: Recent Advances and New Perspectives. International Journal of Molecular Sciences. 21 (6). 1–18. doi: 10.3390/ijms21062174.

Pintus, E., Jovičić, M., Kadlec, M., Ros-Santaella, J. L. 2020. Divergent effect of fast- and slow-releasing H_2S donors on boar spermatozoa under oxidative stress. Scientific Reports. 10 (1). 6508. doi: 10.1038/s41598-020-63489-4.

Pintus, E., Kadlec, M., Jovičić, M., Sedmíková, M., Ros-Santaella, J. L. 2018. Aminoguanidine protects boar spermatozoa against the deleterious effects of oxidative stress. Pharmaceutics. 10 (4). doi: 10.3390/pharmaceutics10040212.

animals

MDPI

Article

The Interaction of NO and H_2S in Boar Spermatozoa under Oxidative Stress

Martin Kadlec *, Eliana Pintus and José Luis Ros-Santaella

Department of Veterinary Sciences, Faculty of Agrobiology, Food and Natural Resources, Czech University of Life Sciences Prague, Kamýcká 129, 165 00 Prague, Czech Republic; pintus@af.czu.cz (E.P.); ros-santaella@ftz.czu.cz (J.L.R.-S.)

* Correspondence: kadlecmartin@af.czu.cz

Simple Summary: The most recent experiments performed on somatic cells describe the interaction of nitric oxide (NO) and hydrogen sulfide (H_2S) on various levels. In male gametes, these two gasotransmitters have been studied individually up until today. Both NO and H_2S participate in crucial sperm structural and functional changes before and after ejaculation. Moreover, the two gasotransmitters can augment or mitigate the negative impact of oxidative stress, depending on the concentration. Oxidative stress is a concomitant condition to various male reproduction disorders. In this experiment, we investigated in vitro the simultaneous application of NO and H_2S donors, which was compared to single-donor application (NO or H_2S) at 100 nM concentrations in boar spermatozoa under oxidative stress. The evaluation of sperm qualitative traits revealed a positive effect of the combination of the two donors in DD treatment on progressive motility and plasma membrane integrity compared to the control sample under oxidative stress (CtrOX). The results of this experiment indicate that the combination of NO and H_2S donors exceeds the effect of single-donor application under given conditions. In conclusion, our research indicates the importance of gasotransmitter interaction in male gametes.

Abstract: Various recent studies dedicated to the role of nitric oxide (NO) and hydrogen sulfide (H_2S) in somatic cells provide evidence for an interaction of the two gasotransmitters. In the case of male gametes, only the action of a single donor of each gasotransmitter has been investigated up until today. It has been demonstrated that, at low concentrations, both gasotransmitters alone exert a positive effect on sperm quality parameters. Moreover, the activity of gaseous cellular messengers may be affected by the presence of oxidative stress, an underlying condition of several male reproductive disorders. In this study, we explored the effect of the combination of two donors SNP and NaHS (NO and H_2S donors, respectively) on boar spermatozoa under oxidative stress. We applied NaHS, SNP, and their combination (DD) at 100 nM concentration in boar spermatozoa samples treated with Fe^{2+}/ascorbate system. After 90 min of incubation at 38 °C, we have observed that progressive motility (PMot) and plasma membrane integrity (PMI) were improved ($p < 0.05$) in DD treatment compared to the Ctr sample under oxidative stress (CtrOX). Moreover, the PMot of DD treatment was higher ($p < 0.05$) than that of NaHS. Similar to NaHS, SNP treatment did not overcome the PMot and PMI of CtrOX. In conclusion, for the first time, we provide evidence that the combination of SNP and NaHS surmounts the effect of single-donor application in terms of PMot and PMI in porcine spermatozoa under oxidative stress.

Keywords: gasotransmitter interaction; hydrogen sulfide; nitric oxide; oxidative stress; boar spermatozoa

Citation: Kadlec, M.; Pintus, E.; Ros-Santaella, J.L. The Interaction of NO and H_2S in Boar Spermatozoa under Oxidative Stress. *Animals* **2022**, *12*, 602. https://doi.org/10.3390/ani12050602

Academic Editor: Rodrigo Manjarin

Received: 3 January 2022
Accepted: 23 February 2022
Published: 28 February 2022

1. Introduction

The importance of nitric oxide (NO) and hydrogen sulfide (H_2S) in male reproduction has been widely recognized [1–5]. Together with carbon monoxide (CO), the members of the gasotransmitter family participate not only in spermatogenesis, but also in epididymal

sperm maturation [6,7]. The CO, NO, and H_2S represent the main widely recognized signaling gaseous molecules [8], although other potential gasotransmitters are emerging (e.g., ammonia, methane, or hydrogen) [9]. Unlike the other gasotransmitters, the role of NO in sperm maturation is more controversial, as the elevated concentrations of NO resulting from abundant pathological conditions (e.g., inflammation or varicocele) are connected with decreased semen quality [10,11]. Both gasotransmitters, NO and H_2S, participate in crucial sperm changes that occur prior to fertilization [6]. Apart from tyrosine phosphorylation, the NO is known to activate sGC, which produces cGMP, and thus increases sperm motility [6]. Moreover, it can also protect membrane from lipid peroxidation by increasing the ratio to O_2^- [12]. In the case of H_2S, the activation of mitogen-activated protein kinases (MAPK) [13], PI3K/Akt pathway [14], and mitochondria [15] is associated with increased sperm motility. Moreover, H_2S is known to increase antioxidant capacity [4] through activation of superoxide dismutase (SOD) [15]. Regarding both gasotransmitters (NO and H_2S), the effect is determined by the type of donor and its concentration that is used. Additionally, the effect can also be affected by the presence of other reactive species (RS), which may result in the formation of more reactive substances or substances with increased signaling potential [16,17]. Therefore, RS are carefully controlled by cellular mechanisms, preventing overaccumulation and oxidative stress [18].

Oxidative stress is an underlying concomitant condition to several male reproductive disorders, in which high levels of reactive species cause sperm dysfunction (e.g., decreased sperm motility, impaired membrane and DNA integrity, increased lipid peroxidation, and infertility [19]. Particularly, sperm cells are highly sensitive to supraphysiological levels of RS due to the high content of polyunsaturated fatty acids in their membranes and their limited antioxidant defense [20]. Current RS classification includes reactive oxygen species (ROS), reactive nitrogen species (RNS), and reactive sulfur species (RSS), whereof the last two named groups also involve both gasotransmitters, NO and H_2S. As an example, in a recent study by Zhang et al. [21], the negative impact of elevated concentrations of H_2S on boar sperm motility was observed. Moreover, the presence of oxidative stress can potentiate the negative impact of high gasotransmitter levels, such as in the case of NO [6], which forms peroxynitrite that inhibits mitochondrial activity [22], reduces sperm motility [23], and increases lipid peroxidation of boar sperm membrane [24]. Thus, the effect of the gasotransmitter on sperm functionality and structural integrity depends not only on its concentration, but also on the specific cellular microenvironment and the presence of other RS. Notwithstanding the recent advances in the study of gasotransmitters and male germ cells under oxidative stress, many questions about the complex interactions and relations remain unanswered.

Most of the information available about these two gasotransmitters and their involvement in the cellular microenvironment originates from the study of somatic cells [25–27]. In murine myocardial cells, the application of H_2S leads to the activation of eNOS, an enzyme responsible for NO production [28], which is also present in boar sperm cells, influencing its function [29]. Both NO and H_2S share signaling targets, such as the MAPK pathway [13,30], which participates in sperm capacitation [31]. Other common signaling targets represent ion channels (Ca^{2+}, K^+) involved in crucial sperm processes, starting with sperm maturation and ending with oocyte fertilization [32]. Lastly, there seems to be important direct interaction between NO and H_2S, which contributes to the formation of other signaling molecules with increased signaling potential, such as the nitroxyl radical (HNO) [33]. Among the metabolites resulting from NO and H_2S interaction, such as nitrosothiols and thionitrous acid [33], the HNO stands out as a potentially relevant molecule for sperm biology concerning oxidative stress [5]. Nitroxyl is formed within seconds after the mixture of NO and H_2S donors [34] and seems to mimic the action of NO [35]. Moreover, HNO has been proven to also act as an antioxidant agent with the ability to reduce lipid peroxidation of the plasma membrane in the yeast model [36]. Concerning the signaling targets of each gasotransmitter, the most recent experiments dedicated to somatic cells provide solid evidence for the interaction between NO and H_2S [37,38], yet this phenomenon was not

studied in sperm cells to date. Recently, we reviewed [5] the complex interaction of these two gasotransmitters in somatic cells and the potential implications for sperm cell biology. Based on the growing evidence of the interaction between NO and H_2S in somatic cells, we decided to test the hypothesis of whether the simultaneous application of NO and H_2S donors can potentiate the effect compared to the single-donor application in boar sperm cells exposed to oxidative stress.

2. Materials and Methods

Reagents were purchased from Sigma-Aldrich (Prague, Czech Republic) unless otherwise indicated.

2.1. Sample Collection and Experimental Design

Artificial insemination doses from 18 boars of different breeds were purchased from a pig breeding company. Sperm-rich fractions were collected by the gloved-hand method, diluted with Solusem® extender (AIM Worldwide, Vught, The Netherlands; pH ≈ 7), and transported to the laboratory at 17 °C.

Firstly, the morphology was assessed in the suspension of PBS with glutaraldehyde at 2.5% (*v*/*v*) concentration, and only samples with morphologically normal sperm (>75%) were used for the experiment. Sperm samples from three boars were pooled to reduce the effect of male variability and were centrifuged at 167× *g* for 3 min at 17 °C to remove debris and dead sperm cells. The sperm concentration was then checked by using a Bürker chamber, adjusted to 20 × 10^6 spermatozoa/mL with Solusem®.

Sperm samples were then randomly split into five microcentrifuge tubes (certified free of DNA, DNase, RNase, and endotoxins (pyrogens); material: virgin polypropylene; volume: 2 mL; Neptune Scientific, San Diego, CA, USA): Ctr (control sample without oxidative stress) and CtrOX (control sample under oxidative stress). The remaining three tubes were submitted to oxidative stress: NO donor (SNP 100 nM), H_2S donor (NaHS 100 nM), and their combination (SNP + NaHS 100 nM; duo-donor = DD). All the chemical supplements were diluted in PBS and freshly prepared before the start of each replicate and exposed to light. Oxidative stress was induced by adding a solution of 0.05 mM $FeSO_4$ and 0.5 mM sodium ascorbate (Fe^{2+}/ascorbate) to the sperm samples. The donors were added to the samples at first and, after approximately 3 min, the oxidative stress was then induced in all samples except Ctr. Sperm analyses were performed after 20 min of incubation for the Ctr sample only and after 90 min of incubation at 38 °C in a water bath for all samples. The experiment was replicated six times with six independent semen pools.

Sperm motility was evaluated using CASA (NIS-Elements; Nikon, Tokyo, Japan, and Laboratory Imaging, Prague, Czech Republic). A prewarmed (38 °C) Spermtrack chamber (PROiSER R + D S.L., Paterna, Spain; chamber depth: 20 µm) was loaded with 5 µL of a sample. A total of 10 sperm kinetic parameters were obtained by analyzing six random fields: total motility (TMot, %), progressive motility (PMot, %), average path velocity (VAP, µm/s), curvilinear velocity (VCL, µm/s), straight-line velocity (VSL, µm/s), the amplitude of lateral head displacement (ALH, µm), beat-cross frequency (BCF, Hz), linearity (LIN, VSL/VCL, %), straightness (STR, VSL/VAP, %), and wobble (WOB, VAP/VCL, %). The settings parameters were as follows: frames per second, 60; minimum frames acquired per sperm track, 31; VAP ≥ 10 µm/s to classify a spermatozoon as motile, STR ≥ 80% to classify a spermatozoon as progressive. A minimum of 200 sperm cells were analyzed for each sample. Sperm motility subpopulations were determined by cluster analysis (see statistical analysis) at 90 min of incubation.

The sperm plasma membrane integrity (PMI) was evaluated as previously described [39]. Aliquots of sperm samples were incubated with carboxyfluorescein diacetate (0.46 mg/mL, *w*/*v*, in dimethyl sulfoxide; DMSO), propidium iodide (0.5 mg/mL, *w*/*v*, in phosphate-buffered saline solution; PBS), and formaldehyde solution (0.3%, *v*/*v*) for 10 min at 38 °C in the dark. Then, 200 spermatozoa were evaluated by using epifluorescence microscopy

(40× objective). The spermatozoa showing green fluorescence over the entire head area were considered to have intact plasma membrane.

Acrosome loss was evaluated according to the protocol previously described [40]. After methanol fixation and double washing with PBS, the samples were incubated with peanut agglutinin–fluorescein isothiocyanate (PNA-FITC; 100 µg/mL, *w*/*v*, in PBS) for 10 min at 38 °C in the dark. Epifluorescence microscopy (40× objective) was used to evaluate 200 spermatozoa, and the cells showing no fluorescence over the acrosome were considered as acrosome-lost spermatozoa.

Lipid peroxidation was assessed with the thiobarbituric acid reactive substances (TBARS) assay, as previously described [41]. At the end of each incubation period, sperm aliquots were collected and stored at −80 °C until analysis. The absorbance of each sample was then measured by spectrophotometry at 532 nm (Libra S22, Biochrom, Harvard Bioscience Company, Cambourne, UK). A standard curve was established by using known concentrations of 1,1,3,3-tetramethoxypropane (MDA). The levels of lipid peroxidation are shown as µmol of MDA per 10^8 spermatozoa. The assay was run in duplicate for each sample. The total antioxidant capacity was determined by spectrophotometry (Libra S22, Biochrom, Harvard Bioscience Company, Cambourne, UK) at 660 nm by using the method described previously [42]. The principle of this assay is based on the antioxidant's capacity to reduce 2,2′-azino-bis (3-et hylbenz-thiazoline-6-sulfonic acid) (ABTS) previously oxidized with H_2O_2. A standard curve was established by using known concentrations of 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox). The total antioxidant capacity (TAC) was expressed as Trolox equivalents (mM). The assay was run in duplicate for each sample.

2.2. Statistical Analysis

Data were analyzed with the statistical program SPSS, version 20 (IBM Inc., Chicago, IL, USA). The generalized linear model (GZLM) was applied to analyze the effects of the NO or H_2S donor and their combination (DD) on the sperm variables. The statistical significance was determined at $p < 0.05$. Data are shown as the mean ± standard deviation. For determining sperm motility subpopulations, we used two kinetic parameters that de-fine sperm average velocity (i.e., VAP) and trajectory linearity (i.e., LIN). The number of clusters was automatically determined by the two-step cluster component using the Euclidean distance measure and Schwarz's Bayesian criterion (BIC). After that, the number of clusters previously obtained was used to set up the K-means cluster analysis by using the iteration and classification method. The Kruskal–Wallis analysis was applied to check for differences in sperm subpopulations among treatments. The Wilcoxon signed-rank test (matched samples) was performed to check the differences between kinetic parameters of the subpopulations.

3. Results

3.1. Sperm Motility

The PMot of CtrOX was significantly decreased compared to the control sample without oxidative stress ($p = 0.048$), as seen in Figure 1. The PMot of DD treatment (61.7%) was the only one that significantly exceeded the PMot of the CtrOX sample (54.3%; $p < 0.05$). Moreover, it was also significantly higher than the PMot of NaHS treatment (54.7%; $p < 0.05$). The NaHS had a tendency of lower PMot than the Ctr sample (61.2%; $p = 0.064$). SNP was the only treatment that did not statistically differ from any other sample at 90 min of incubation time. During the incubation of all samples, the value of VSL, LIN, and STR increased, which resulted in more rectilinear trajectories compared to the Ctr sample at 20 min of incubation.

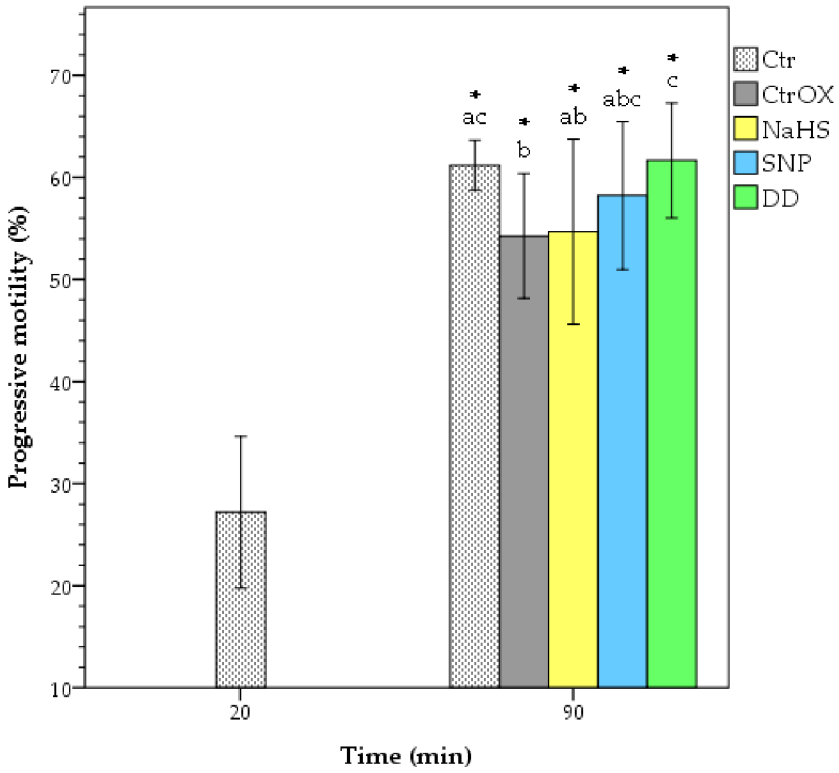


Figure 1. Progressive motility of boar spermatozoa under oxidative stress. Spermatozoa with straightness ≥ 80% were selected as progressive. Different letters indicate significant differences ($p < 0.05$) among treatments at 90 min of incubation. The asterisks indicate significant differences ($p < 0.05$) of samples compared to Ctr at 20 min of incubation. CTR = control; CtrOX = control under oxidative stress; NaHS = 100 nM; SNP = 100 nM; DD = SNP 100 nM + NaHS 100 nM. Data are shown as mean ± SD of 6 replicates.

The CtrOX had significantly reduced total motility (TMot) compared to Ctr sample ($p = 0.038$). In continuation, all donor samples under oxidative stress had comparable TMot to control sample without oxidative stress ($p > 0.05$), although TMot of NaHS treatment tended to be lower than the in Ctr sample ($p = 0.058$). A significant difference ($p < 0.05$) among treatments was seen between BCF of DD treatment, which was higher than the one of NaHS. Interestingly, the BCF of the DD sample tended to be higher than the Ctr sample ($p = 0.076$). Complete kinetic parameters are shown in Table 1. Cluster analysis rendered two sperm subpopulations that, based on their kinetics, were classified as rapid progressive (Sp1) and slow nonprogressive (Sp2). Sperm subpopulations differed among them in all of the sperm kinetic parameters (Table S1). However, there were no significant differences between treatments ($p > 0.05$; Figure S1). Interestingly, NaHS showed the smallest Sp1 (38.2%), which was 1% smaller than Sp1 of CtrOX (39.2%). SNP treatment contained 42.9% of rapid progressive spermatozoa (Sp1). Yet, the Sp1 was most represented in the DD sample (44.6%) that also exceeded the Sp1 of Ctr (43.8%; $p > 0.05$).

Table 1. The effect of NaHS, SNP, and their combination (DD) on kinetic parameters of boar spermatozoa under oxidative stress.

Treatment	Time (min)	TMot (%)	VAP (μm/s)	VCL (μm/s)	VSL (μm/s)	ALH (μm)	BCF (Hz)	LIN (%)	STR (%)	WOB (%)
Ctr	20	65.9 ± 8.2	40.1 ± 6.1	95.2 ± 8.2	24.9 ± 4.7	3.0 ± 0.3	11.4 ± 0.7	28.4 ± 4.7	66.6 ± 6.0	40.8 ± 3.6
Ctr	90	66.8 ± 2.9 ^{a}	40.5 ± 4.3	71.7 ± 6.9 *	36.7 ± 4.1 *	2.8 ± 0.3	14.0 ± 0.5 ^{ab,*}	50.0 ± 2.8 *	88.4 ± 1.6 *	54.9 ± 2.4 *
CtrOX	90	55.7 ± 11.2 ^{b}	41.0 ± 8.4	77.5 ± 17.5 *	34.4 ± 7.1 *	2.7 ± 0.6	14.2 ± 0.8 ^{ab,*}	48.8 ± 7.6 *	85.4 ± 6.3 *	55.3 ± 5.1 *
NaHS	90	56.7 ± 13.1 ^{ab}	38.8 ± 4.8	71.0 ± 13.7 *	33.8 ± 3.7 *	2.6 ± 0.4	13.8 ± 0.5 ^{a,*}	49.5 ± 8.2 *	86.7 ± 7.9 *	55.2 ± 5.0 *
SNP	90	59.4 ± 12.2 ^{ab}	40.9 ± 5.9	72.2 ± 11.3 *	36.4 ± 4.8 *	2.7 ± 0.4	14.3 ± 1.0 ^{ab,*}	51.9 ± 5.5 *	88.7 ± 4.4 *	57.1 ± 4.3 *
DD	90	60.5 ± 9.8 ^{ab}	40.2 ± 6.1	70.0 ± 15.5 *	36.2 ± 4.8 *	2.7 ± 0.5	14.7 ± 1.1 ^{b,*}	53.4 ± 7.2 *	89.7 ± 5.6 *	58.2 ± 5.0 *

Different letters indicate significant differences ($p < 0.05$) among treatments at 90 min of incubation. The asterisks indicate significant differences ($p < 0.05$) of samples compared to Ctr at 20 min incubation. CTR = control; CtrOX = control under oxidative stress; NaHS 100 nM; SNP = 100 nM; DD = SNP 100 nM + NaHS 100 nM. TMot: total motility; VAP: average path velocity; VCL: curvilinear velocity; VSL: straight-line velocity; ALH: amplitude of lateral head displacement; BCF: beat-cross frequency; LIN: linearity (VSL/VCL); STR: straightness (VSL/VAP); WOB: wobble (VAP/VCL). Data are shown as the mean ± SD of 6 replicates.

3.2. Plasma Membrane Integrity and Lipid Peroxidation

There was no difference in PMI between Ctr and CtrOX at 90 min of incubation time (Figure 2). The treatment DD showed a higher PMI in comparison to the CtrOX ($p < 0.05$). Although not significant ($p > 0.05$), only in DD treatment there was a higher PMI percentage than in the Ctr sample at 90 min of incubation. Moreover, DD treatment was the only one that did not show significant differences ($p > 0.05$) with the Ctr sample at 20 min of incubation. Yet, there was a decrease in PMI of the Ctr sample during incubation (89.7% vs. 81.3% respectively; $p < 0.05$). Moreover, there was a tendency in DD treatment to have higher PMI than treatments with NaHS and SNP alone ($p = 0.076$ and $p = 0.067$ respectively). All samples under oxidative stress showed higher levels of lipid peroxidation (LP; $p < 0.05$) than the control sample without oxidative stress (Figure 3). No significant differences ($p > 0.05$) among treatments under oxidative stress were found in terms of LP. The levels of LP in the Ctr sample did not change during incubation time ($p > 0.05$).

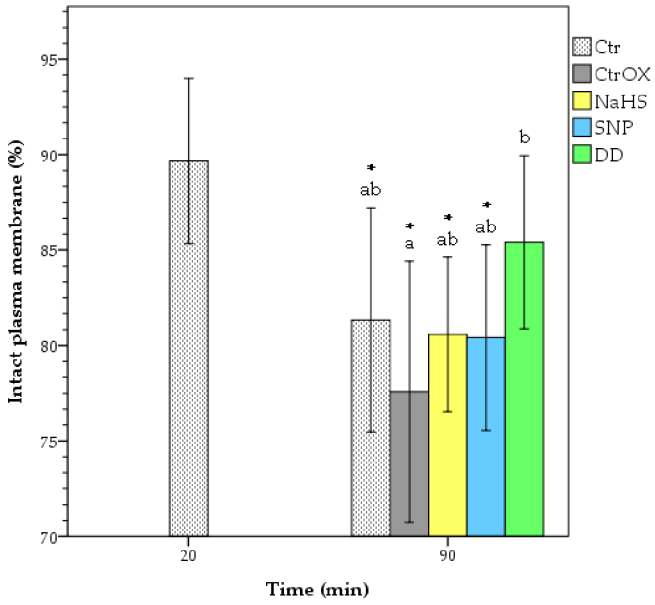


Figure 2. Percentage of membrane intact boar spermatozoa under oxidative stress. Different letters indicate significant differences ($p < 0.05$) between treatments at 90 min of incubation. The asterisks indicate significant differences ($p < 0.05$) of samples compared to Ctr at 20 min incubation. CTR = control; CtrOX = control under oxidative stress; NaHS = 100 nM; SNP = 100 nM; DD = SNP 100 nM + NaHS 100 nM. Data are shown as mean ± SD of 6 replicates.

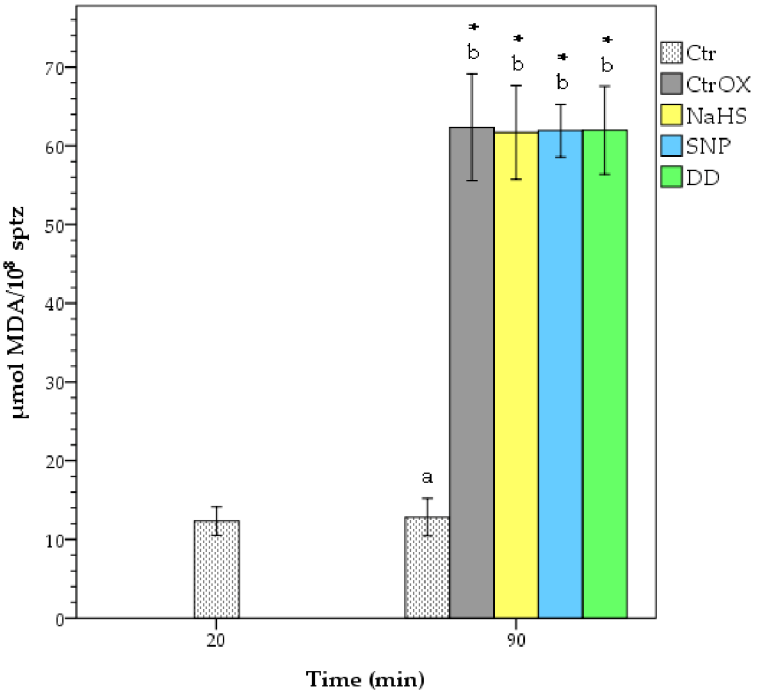


Figure 3. Levels of lipid peroxidation of boar spermatozoa under oxidative stress. Different letters indicate significant differences ($p < 0.05$) between treatments at 90 min of incubation. The asterisks indicate significant differences ($p < 0.05$) of samples compared to Ctr at 20 min incubation. CTR = control; CtrOX = control under oxidative stress; NaHS = 100 nM; SNP = 100 nM; DD = SNP 100 nM + NaHS 100 nM. Data are shown as mean ± SD of 6 replicates.

3.3. Acrosome Integrity

No significant differences were found among the samples. Interestingly, the treatment SNP had the lowest percentage of intact acrosome and tended to be lower than the Ctr sample ($p = 0.085$), and then DD treatment ($p = 0.075$). Moreover, the SNP treatment had the highest standard deviation, which was more than 3 times higher than in any other treatment (Table 2).

Table 2. The effect of NaHS, SNP, and their combination (DD) on percentage of acrosome intact boar spermatozoa under oxidative stress.

Treatment	Time (min)	Intact Acrosome (%)
Ctr	20	98.0 ± 1.4
Ctr	90	97.8 ± 2.0
CtrOX	90	96.2 ± 1.2
NaHS	90	97.1 ± 1.6
SNP	90	95.5 ± 5.3
DD	90	97.9 ± 1.4

CTR = control; CtrOX = control under oxidative stress; NaHS = 100 nM; SNP = 100 nM; DD = SNP 100 nM + NaHS 100 nM. Data are shown as mean ± SD of 6 replicates.

3.4. Total Antioxidant Capacity

As shown in Table 3, the TAC was significantly reduced in all treatments under oxidative stress compared to the Ctr sample at 90 min of incubation ($p < 0.05$). No significant differences were observed between the treatments under oxidative stress. TAC of the Ctr sample without oxidative stress decreased during the incubation ($p < 0.05$).

Table 3. The effect of NaHS, SNP, and their combination (DD) on the levels of total antioxidant capacity in different samples of boar spermatozoa under oxidative stress.

Treatment	Time (min)	Total Antioxidant Capacity (mM)
Ctr	20	0.60 ± 0.06
Ctr	90	* 0.54 ± 0.06 [a]
CtrOX	90	* 0.43 ± 0.04 [b]
NaHS	90	* 0.42 ± 0.04 [b]
SNP	90	* 0.41 ± 0.04 [b]
DD	90	* 0.42 ± 0.05 [b]

Different letters indicate significant differences ($p < 0.05$) among treatments at 90 min of incubation. The asterisks indicate significant differences ($p < 0.05$) of samples compared to Ctr at 20 min incubation. CTR = control; CtrOX = control under oxidative stress; NaHS = 100 nM; SNP = 100 nM; DD = SNP 100 nM + NaHS 100 nM. Data are shown as mean ± SD of 6 replicates.

4. Discussion

In the present study, we investigated the differences in boar sperm quality traits in samples under oxidative stress supplemented with NO donor (SNP 100 nM), H_2S donor (NaHS 100 nM), and their combination (SNP + NaHS 100 nM; DD). Our results support the hypothesis that there is a synergy between NO and H_2S protecting membrane integrity and increasing progressive motility in boar spermatozoa under oxidative stress. This is the first study performed on male gametes testing the simultaneous NO and H_2S donor application. Several combinations of NO and H_2S donors have been tested in somatic cells to date and this is the first study that has tested the combination of NO/H_2S donors on sperm cells to the best of our knowledge. In extensive research by Yong et al. [43,44], the combination of SNP and NaHS was tested in the cardiovascular system. In the study from 2010 [43], the author collective established the ideal ratio between the two above-mentioned donors to be 1:1 in terms of the effectiveness of cardiomyocyte shortening. Moreover, Yong et al. [43] indirectly demonstrated that HNO is formed as the result of the two donors' combination using HNO scavengers. NaHS together with Na_2S is the most common sulfide salt used in a biological system as H_2S donors. The two sulfide salts can substitute each other in terms of main characteristics, both being fast and direct donors releasing relatively high amounts of H_2S in a short period of time [45]. In a study from 2014, Eberhardt et al. [34] tested the combination of NO and H_2S donors (DEA NONOate, Na_2S, respectively) in the neurovascular system. Using an HNO-selective electrode, they have demonstrated that the mixture of NO and H_2S (Na_2S donor) leads to immediate HNO formation. The peak of HNO formation was observed after 1 min of the NO and Na_2S mixture [34]. Based upon these studies and our preliminary experiments, we decided to use the combination of two fast-releasing NO and H_2S donors, SNP and NaHS, and established the ratio and concentration at 100 nM:100 nM. The concentration of each donor used was also set considering our previous studies [4,46] that indicated the effective concentrations of each NO and H_2S donor in boar spermatozoa. Moreover, we bore in mind the estimated physiological concentrations, which, in general, are within the nM range for both gasotransmitters [47]. To artificially induce oxidative stress, we used Fe^{2+}/ascorbate as a ROS-generating system. This model is suitable for studies of lipid peroxidation [41], which belongs to the main causes of sperm membrane degradation [48]. The induction of oxidative stress in our study results in decreased motility (TMot and PMot) and TAC, and increased LP in the CtrOX sample compared to Ctr. Comparing the treatments containing donors alone and their combination, some interesting differences were observed in sperm motility and PMI.

The DD treatment with two donors combined show significantly higher PMI and PMot than CtrOX, and also preserved PMI compared to Ctr at 20 min. Moreover, DD treatment exceeded NaHS treatment in terms of PMot. Interestingly, SNP treatment exerted a mild effect with TMot and PMot values in between the ones of NaHS and DD, respectively. Moreover, SNP was characterized by a higher standard deviation in the case of acrosome

integrity compared to the rest of the treatments. Perhaps the formation of more reactive and pro-oxidative molecules, such as peroxynitrite, could explain the reduced positive effect on plasma membrane integrity and sperm motility, and also the partial acrosome destabilization when compared to DD treatment. On the other hand, NaHS treatment showed no effect and tended to have several lower sperm quality traits (e.g., TM) in comparison to Ctr. We speculate that, in such a low dose, only a partial increase in activity of SOD occurred, depleting the H_2S pool. This could result in mild protection of plasma membrane integrity compared to CtrOX, although no increase in sperm motility was seen. Taken together, these results indicate that the two donors combined more resemble the action of SNP alone rather than NaHS in the presence of oxidative stress. Yet, the combination of both donors seems to be more efficient than SNP alone, as seen in the case of progressive motility. This leads to speculation that the interaction of the two donors results in the formation of a more potent metabolite that mimics the action of NO (Figure S2). Indeed, various studies state that the interaction between H_2S and NO leads to the formation of HNO, which mimics the action of NO [33,49]. HNO resulting from NaHS and NO interaction seems to be a more potent signal transductor than its precursor, NO, in the vascular system [33]. Yet, this hypothesis remains to be tested. In a previous study performed on human spermatozoa [13], NaHS donor used at 5 μM concentration under in vitro conditions impaired sperm motility. Accordingly, in our previous study [4], Na_2S donor had a positive effect at the lowest concentration (3 μM) on TMot but impaired the quality of boar spermatozoa at higher concentrations. In this study, NaHS did not affect boar spermatozoa at 100 nM concentration. These results indicate that the doses with a positive effect on sperm quality are at very low micromolar concentrations in the case of fast-releasing donors of H_2S applied to boar spermatozoa in vitro and under oxidative stress. Overall, we observed higher values of various sperm quality traits (TMot, PMot, PMI) in samples treated with SNP 100 nM compared to the control sample with oxidative stress, although not statistically significant. In another study performed by Hellstorm et al. [12], thawed human spermatozoa were treated with SNP at 50 and 100 nM concentration, and positive effects on sperm viability (eosin staining) and motility and reduction in lipid peroxidation were observed. The difference in the results could be attributed to the fact that, in our study, the positive effect might be masked by the presence of additionally induced oxidative stress. The reaction of NO with ROS results in the formation of peroxynitrite, which impairs sperm quality traits and the state of lipid peroxidation in boar spermatozoa at micromolar concentrations [24]. An interesting observation was made concerning the kinetic parameters of the CASA analysis. The sample DD had a significantly higher value of beat-cross frequency (BCF) than the sample NaHS, indicating a difference in motility pattern. The BCF parameter is suggested as one of the predictive factors of boar fertility and insemination success [50].

As stated above, an increased PMI was observed in the treatment containing both donors (DD), which was the only one that significantly exceeded the PMI of the CtrOX sample. Moreover, the DD treatment did not differ from the control sample without oxidative stress at 20 min of incubation. The percentage of spermatozoa with an intact membrane is associated with increased fertilization potential in boar [51–53], and other mammals as well [54,55]. Similarly to the plasma membrane, acrosome integrity is essential for the proper function of a sperm cell during fertilization in mammals [56]. Despite no statistical significance, an interesting observation concerning acrosome integrity of SNP treatment was made, since the standard deviation was more than doubled in comparison to all other treatments. Numerous studies dedicated to the effect of NO on spermatozoa demonstrated its involvement in acrosomal reaction [1,57,58]. The SNP at μM concentrations triggers acrosome reaction in human spermatozoa [59,60] and boar spermatozoa [61,62]. Our results indicate the possibility that partial acrosome membrane destabilization occurred in samples treated with 100 nM SNP. Perhaps this occurred due to conversion of NO in the presence of oxidative stress to $ONOO^-$, which is a potent acrosomal reaction inducer.

5. Conclusions

For the first time, the effect of simultaneous application of NO and H_2S donors has been tested in boar spermatozoa exposed to oxidative stress. Our results indicate a possible synergy between the two gasotransmitters, increasing progressive motility and protecting plasma membrane integrity. The dual NO and H_2S donor application was the only one that resulted in an increased PMot and PMI compared to CtrOX. Interestingly, SNP treatment was rather similar to DD. On the other hand, NaHS treatment showed impaired PMot compared to DD and converged to lower motility (TMot and PMot) than Ctr. These results indicate the importance and the complexity of gasotransmitter interactions in the male gametes.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/ani12050602/s1, Figure S1: Distribution of subpopulations among treatments at 90 min of incubation; Figure S2. The physiological action of NO and H_2S and the hypothesized action related to observed results; Table S1. Kinetic parameters of different subpopulations

Author Contributions: Conceptualization, M.K.; methodology, M.K., E.P. and J.L.R.-S.; supervision, E.P. and J.L.R.-S.; investigation, M.K., E.P. and J.L.R.-S.; formal analysis and data curation, M.K., E.P. and J.L.R.-S.; writing—original draft preparation, M.K.; writing—review and editing, M.K., E.P. and J.L.R.-S. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study are available in Supplementary Materials (Dataset S1 and S2).

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Buzadzic, B.; Vucetic, M.; Jankovic, A.; Stancic, A.; Korac, A.; Korac, B.; Otasevic, V. New insights into male (in)fertility: The importance of NO. *Br. J. Pharmacol.* **2015**, *172*, 1455–1467. [CrossRef] [PubMed]
2. Toor, J.S.; Sikka, S.C. *Human Spermatozoa and Interactions with Oxidative Stress*; Elsevier Inc.: Amsterdam, The Netherlands, 2019; Volume 2, ISBN 9780128125014.
3. Di Villa Bianca, R.D.E.; Sorrentino, R.; Maffia, P.; Mirone, V.; Imbimbo, C.; Fusco, F.; De Palma, R.; Ignarro, L.J.; Cirino, G. Hydrogen sulfide as a mediator of human corpus cavernosum smooth-muscle relaxation. *Proc. Natl. Acad. Sci. USA.* **2009**, *106*, 4513–4518. [CrossRef] [PubMed]
4. Pintus, E.; Jovičić, M.; Kadlec, M.; Ros-Santaella, J.L. Divergent effect of fast- and slow-releasing H_2S donors on boar spermatozoa under oxidative stress. *Sci. Rep.* **2020**, *10*, 6508. [CrossRef]
5. Kadlec, M.; Ros-Santaella, J.L.; Pintus, E. The roles of no and h2s in sperm biology: Recent advances and new perspectives. *Int. J. Mol. Sci.* **2020**, *21*, 2174. [CrossRef]
6. Otasevic, V.; Stancic, A.; Korac, A.; Jankovic, A.; Korac, B. Reactive oxygen, nitrogen, and sulfur species in human male fertility. A crossroad of cellular signaling and pathology. *BioFactors* **2020**, *46*, 206–219. [CrossRef]
7. Lee, N.P.Y.; Cheng, C.Y. Nitric oxide and cyclic nucleotides: Their roles in junction dynamics and spermatogenesis. *Oxid. Med. Cell. Longev.* **2008**, *1*, 25–32. [CrossRef] [PubMed]
8. Wang, R. Two's company, three's a crowd: Can H_2S be the third endogenous gaseous transmitter? *FASEB J.* **2002**, *16*, 1792–1798. [CrossRef] [PubMed]
9. Wang, R. Gasotransmitters: Growing pains and joys. *Trends Biochem. Sci.* **2014**, *39*, 227–232. [CrossRef]
10. Bahmanzadeh, M.; Abolhassani, F.; Amidi, F.; Ejtemaiemehr, S.H.; Salehi, M.; Abbasi, M. The effects of nitric oxide synthase inhibitor (L-NAME) on epididymal sperm count, motility, and morphology in varicocelized rat. *Mol. Cell. Neurosci.* **2008**, *16*, 23–38.
11. Türkyilmaz, Z.; Gülen, Ş.; Sönmez, K.; Karabulut, R.; Dinçer, S.; Başaklar, A.C.; Kale, N. Increased nitric oxide is accompanied by lipid oxidation in adolescent varicocele. *Int. J. Androl.* **2004**, *27*, 183–187. [CrossRef]
12. Hellstrom, W.J.G.; Bell, M.; Wang, R.; Sikka, S.C. Effect of sodium nitroprusside on sperm motility, viability, and lipid peroxidation. *Fertil. Steril.* **1994**, *61*, 1117–1122. [CrossRef]

13. Wang, J.; Wang, W.; Li, S.; Han, Y.; Zhang, P.; Meng, G.; Xiao, Y.; Xie, L.; Wang, X.; Sha, J.; et al. Hydrogen Sulfide As a Potential Target in Preventing Spermatogenic Failure and Testicular Dysfunction. *Antioxid. Redox Signal.* **2018**, *28*, 1447–1462. [CrossRef]
14. Xia, Y.; Ning, J.; Cheng, F.; Yu, W.; Rao, T.; Ruan, Y.; Yuan, R.; Du, Y. GYY4137 a H_2S donor, attenuates ipsilateral epididymis injury in experimentally varicocele-induced rats via activation of the PI3K/Akt pathway. *Iran. J. Basic Med. Sci.* **2019**, *22*, 729–735. [CrossRef]
15. Li, G.; Xie, Z.Z.; Chua, J.M.W.; Wong, P.C.; Bian, J. Hydrogen sulfide protects testicular germ cells against heat-induced injury. *Nitric Oxide-Biol. Chem.* **2015**, *46*, 165–171. [CrossRef] [PubMed]
16. Hancock, J.T.; Whiteman, M. Hydrogen sulfide signaling: Interactions with nitric oxide and reactive oxygen species. *Ann. N. Y. Acad. Sci.* **2016**, *1365*, 5–14. [CrossRef] [PubMed]
17. Mishanina, T.V.; Libiad, M.; Banerjee, R. Biogenesis of reactive sulfur species for signaling by hydrogen sulfide oxidation pathways. *Nat. Chem. Biol.* **2015**, *11*, 457–464. [CrossRef] [PubMed]
18. O'Flaherty, C.; Matsushita-Fournier, D. Reactive oxygen species and protein modifications in spermatozoa. *Biol. Reprod.* **2017**, *97*, 577–585. [CrossRef] [PubMed]
19. Aitken, R.J.; Jones, K.T.; Robertson, S.A. Reactive oxygen species and sperm function-in sickness and in health. *J. Androl.* **2012**, *33*, 1096–1106. [CrossRef]
20. Aitken, R.J.; Gibb, Z.; Baker, M.A.; Drevet, J.; Gharagozloo, P. Causes and consequences of oxidative stress in Spermatozoa. *Reprod. Fertil. Dev.* **2016**, *28*, 1–10. [CrossRef]
21. Zhang, W.; Zhao, Y.; Zhang, P.; Hao, Y.; Yu, S.; Min, L.; Li, L.; Ma, D.; Chen, L.; Yi, B.; et al. Decrease in male mouse fertility by hydrogen sulfide and/or ammonia can Be inheritable. *Chemosphere* **2018**, *194*, 147–157. [CrossRef] [PubMed]
22. Radi, R. Oxygen radicals, nitric oxide, and peroxynitrite: Redox pathways in molecular medicine. *Proc. Natl. Acad. Sci. USA* **2018**, *115*, 5839–5848. [CrossRef] [PubMed]
23. Uribe, P.; Boguen, R.; Treulen, F.; Sänchez, R.; Villegas, J.V. Peroxynitrite-mediated nitrosative stress decreases motility and mitochondrial membrane potential in human spermatozoa. *Mol. Hum. Reprod.* **2014**, *21*, 237–243. [CrossRef] [PubMed]
24. Serrano, R.; Garrido, N.; Céspedes, J.A.; González-Fernández, L.; García-Marín, L.J.; Bragado, M.J. Molecular Mechanisms Involved in the Impairment of Boar Sperm Motility by Peroxynitrite-Induced Nitrosative Stress. *Int. J. Mol. Sci.* **2020**, *21*, 1208. [CrossRef] [PubMed]
25. Cirino, G.; Vellecco, V.; Bucci, M. Nitric oxide and hydrogen sulfide: The gasotransmitter paradigm of the vascular system. *Br. J. Pharmacol.* **2017**, *174*, 4021–4031. [CrossRef] [PubMed]
26. Panthi, S.; Manandhar, S.; Gautam, K. Hydrogen sulfide, nitric oxide, and neurodegenerative disorders. *Transl. Neurodegener.* **2018**, *7*, 3. [CrossRef] [PubMed]
27. Wallace, J.L.; Ianaro, A.; de Nucci, G. Gaseous Mediators in Gastrointestinal Mucosal Defense and Injury. *Dig. Dis. Sci.* **2017**, *62*, 2223–2230. [CrossRef]
28. King, A.L.; Polhemus, D.J.; Bhushan, S.; Otsuka, H.; Kondo, K.; Nicholson, C.K.; Bradley, J.M.; Islam, K.N.; Calvert, J.W.; Tao, Y.X.; et al. Hydrogen sulfide cytoprotective signaling is endothelial nitric oxide synthase-nitric oxide dependent. *Proc. Natl. Acad. Sci. USA* **2014**, *111*, 3182–3187. [CrossRef]
29. Staicu, F.D.; Lopez-Úbeda, R.; Romero-Aguirregomezcorta, J.; Martínez-Soto, J.C.; Matás Parra, C. Regulation of boar sperm functionality by the nitric oxide synthase/nitric oxide system. *J. Assist. Reprod. Genet.* **2019**, *36*, 1721–1736. [CrossRef]
30. Lee, N.P.Y.; Cheng, C.Y. Nitric Oxide/Nitric Oxide Synthase, Spermatogenesis, and Tight Junction Dynamics. *Biol. Reprod.* **2004**, *70*, 267–276. [CrossRef] [PubMed]
31. Gangwar, D.K.; Atreja, S.K. Signalling Events and Associated Pathways Related to the Mammalian Sperm Capacitation. *Reprod. Domest. Anim.* **2015**, *50*, 705–711. [CrossRef]
32. Kolluru, G.K.; Shen, X.; Yuan, S.; Kevil, C.G. Gasotransmitter heterocellular signaling. *Antioxid. Redox Signal.* **2017**, *26*, 936–960. [CrossRef]
33. Ivanovic-Burmazovic, I.; Filipovic, M.R. Saying NO to H2S: A Story of HNO, HSNO, and SSNO. *Inorg. Chem.* **2019**, *58*, 4039–4051. [CrossRef] [PubMed]
34. Eberhardt, M.; Dux, M.; Namer, B.; Miljkovic, J.; Cordasic, N.; Will, C.; Kichko, T.I.; De La Roche, J.; Fischer, M.; Suárez, S.A.; et al. H_2S and NO cooperatively regulate vascular tone by activating a neuroendocrine HNO-TRPA1-CGRP signalling pathway. *Nat. Commun.* **2014**, *5*, 4381. [CrossRef] [PubMed]
35. Andrews, K.L.; Lumsden, N.G.; Farry, J.; Jefferis, A.M.; Kemp-Harper, B.K.; Chin-Dusting, J.P.F. Nitroxyl: A vasodilator of human vessels that is not susceptible to tolerance. *Clin. Sci.* **2015**, *129*, 179–187. [CrossRef] [PubMed]
36. Lopez, B.E.; Shinyashiki, M.; Han, T.H.; Fukuto, J.M. Antioxidant actions of nitroxyl (HNO). *Free Radic. Biol. Med.* **2007**, *42*, 482–491. [CrossRef] [PubMed]
37. Nagpure, B.V.; Bian, J.S. Interaction of Hydrogen Sulfide with Nitric Oxide in the Cardiovascular System. *Oxid. Med. Cell. Longev.* **2016**, *2016*, 6904327. [CrossRef]
38. Kajimura, M.; Fukuda, R.; Bateman, R.M.; Yamamoto, T.; Suematsu, M. Interactions of multiple gas-transducing systems: Hallmarks and uncertainties of CO, NO, and H_2S Gas Biology. *Antioxid. Redox Signal.* **2010**, *13*, 157–192. [CrossRef]
39. Harrison, R.A.P.; Vickers, S.E. Use of fluorescent probes to assess membrane integrity in mammalian spermatozoa. *J. Reprod. Fertil.* **1990**, *88*, 343–352. [CrossRef]

40. García-Vázquez, F.A.; Hernández-Caravaca, I.; Yánez-Quintana, W.; Matás, C.; Soriano-Úbeda, C.; Izquierdo-Rico, M.J. Morphometry of boar sperm head and flagellum in semen backflow after insemination. *Theriogenology* **2015**, *84*, 566–574. [CrossRef]
41. Brzezinska-Slebodzinska, E.; Slebodzinska, A.B.; B, P.; Wieczorek, G. Antioxidant effect of vitamin E and glutathione on lipid peroxidation in boar semen plasma. *Biol. Trace Elem. Res.* **1995**, *47*, 69–74. [CrossRef]
42. Erel, O. A novel automated direct measurement method for total antioxidant capacity using a new generation, more stable ABTS radical cation. *Clin. Biochem.* **2004**, *37*, 277–285. [CrossRef]
43. Yong, Q.C.; Hu, L.F.; Wang, S.; Huang, D.; Bian, J.S. Hydrogen sulfide interacts with nitric oxide in the heart: Possible involvement of nitroxyl. *Cardiovasc. Res.* **2010**, *88*, 482–491. [CrossRef] [PubMed]
44. Yong, Q.C.; Cheong, J.L.; Hua, F.; Deng, L.W.; Khoo, Y.M.; Lee, H.S.; Perry, A.; Wood, M.; Whiteman, M.; Bian, J.S. Regulation of heart function by endogenous gaseous mediators-crosstalk between nitric oxide and hydrogen sulfide. *Antioxid. Redox Signal.* **2011**, *14*, 2081–2091. [CrossRef]
45. Powell, C.R.; Dillon, K.M.; Matson, J.B. A review of hydrogen sulfide (H_2S) donors: Chemistry and potential therapeutic applications. *Biochem. Pharmacol.* **2018**, *149*, 110–123. [CrossRef] [PubMed]
46. Jovičić, M.; Pintus, E.; Fenclová, T.; Imoník, O.S.; Chmelíková, E.; Ros-Santaella, J.L.; Sedmíková, M. Effect of nitric oxide on boar sperm motility, membrane integrity, and acrosomal status during semen storage. *Pol. J. Vet. Sci.* **2018**, *21*, 73–82. [CrossRef]
47. Cooper, C.E.; Brown, G.C. The inhibition of mitochondrial cytochrome oxidase by the gases carbon monoxide, nitric oxide, hydrogen cyanide and hydrogen sulfide: Chemical mechanism and physiological significance. *J. Bioenerg. Biomembr.* **2008**, *40*, 533–539. [CrossRef] [PubMed]
48. Awda, B.J.; Mackenzie-bell, M.; Buhr, M.M. Reactive Oxygen Species and Boar Sperm Function. *Biol. Reprod.* **2009**, *81*, 553–561. [CrossRef] [PubMed]
49. Cortese-Krott, M.M.; Fernandez, B.O.; Kelm, M.; Butler, A.R.; Feelisch, M. On the chemical biology of the nitrite/sulfide interaction. *Nitric Oxide-Biol. Chem.* **2015**, *46*, 14–24. [CrossRef]
50. Schulze, M.; Ruediger, K.; Mueller, K.; Jung, M.; Well, C.; Reissmann, M. Development of an in vitro index to characterize fertilizing capacity of boar ejaculates. *Anim. Reprod. Sci.* **2013**, *140*, 70–76. [CrossRef]
51. Sutkeviciene, N.; Riskeviciene, V.; Januskauskas, A.; Zilinskas, H.; Andersson, M. Assessment of sperm quality traits in relation to fertility in boar semen. *Acta Vet. Scand.* **2009**, *51*, 53. [CrossRef]
52. Gil, M.A.; Almiñana, C.; Roca, J.; Vázquez, J.M.; Martínez, E.A. Boar semen variability and its effects on IVF efficiency. *Theriogenology* **2008**, *70*, 1260–1268. [CrossRef] [PubMed]
53. Berger, T.; Anderson, D.L.; Penedo, M.C.T. Porcine sperm fertilizing potential in relationship to sperm functional capacities. *Anim. Reprod. Sci.* **1996**, *44*, 231–239. [CrossRef]
54. Brito, L.F.C.; Barth, A.D.; Bilodeau-Goeseels, S.; Panich, P.L.; Kastelic, J.P. Comparison of methods to evaluate the plasmalemma of bovine sperm and their relationship with in vitro fertilization rate. *Theriogenology* **2003**, *60*, 1539–1551. [CrossRef]
55. Ramu, S.; Jeyendran, R.S. The hypo-osmotic swelling test for evaluation of sperm membrane integrity. *Methods Mol. Biol.* **2013**, *927*, 21–25. [CrossRef] [PubMed]
56. Beltrán, C.; Treviño, C.L.; Mata-Martínez, E.; Chávez, J.C.; Sánchez-Cárdenas, C.; Baker, M.; Darszon, A. *Sperm Acrosome Biogenesis and Function during Fertilization*; Advances in Anatomy, Embryology and Cell Biology; Buffone, M.G., Ed.; Springer International Publishing: Cham, Switzerland, 2016; Volume 220, ISBN 978-3-319-30565-3.
57. 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. [CrossRef]
58. Staicu, F.-D.; Parra, C.M. Nitric Oxide: Key Features in Spermatozoa. In *Nitric Oxide Synthase-Simple Enzyme-Complex Roles*; InTech: London, UK, 2017; Volume i, p. 13.
59. Revelli, A.; Costamagna, C.; Moffa, F.; Aldieri, E.; Ochetti, S.; Bosia, A.; Massobrio, M.; Lindblom, B.; Ghigo, D. Signaling pathway of nitric oxide-induced acrosome reaction in human spermatozoa. *Biol. Reprod.* **2001**, *64*, 1708–1712. [CrossRef]
60. 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*, 522–527. [CrossRef]
61. 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. [CrossRef]
62. 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*, 857–864. [CrossRef] [PubMed]

International Journal of
Molecular Sciences

MDPI

Review

The Roles of NO and H_2S in Sperm Biology: Recent Advances and New Perspectives

Martin Kadlec, José Luis Ros-Santaella and Eliana Pintus *

Department of Veterinary Sciences, Faculty of Agrobiology, Food and Natural Resources, Czech University of Life Sciences Prague, Kamýcká 129, 165 00 Praha 6-Suchdol, Czech Republic; kadlecmartin@af.czu.cz (M.K.); ros-santaella@ftz.czu.cz (J.L.R.-S.)

* Correspondence: pintus@af.czu.cz; Tel.: +420-224-382-939

Received: 5 February 2020; Accepted: 18 March 2020; Published: 21 March 2020

Abstract: After being historically considered as noxious agents, nitric oxide (NO) and hydrogen sulfide (H_2S) are now listed as gasotransmitters, gaseous molecules that play a key role in a variety of cellular functions. Both NO and H_2S are endogenously produced, enzymatically or non-enzymatically, and interact with each other in a range of cells and tissues. In spite of the great advances achieved in recent decades in other biological systems, knowledge about H_2S function and interactions with NO in sperm biology is in its infancy. Here, we aim to provide an update on the importance of these molecules in the physiology of the male gamete. Special emphasis is given to the most recent advances in the metabolism, mechanisms of action, and effects (both physiological and pathophysiological) of these gasotransmitters. This manuscript also illustrates the physiological implications of NO and H_2S observed in other cell types, which might be important for sperm function. The relevance of these gasotransmitters to several signaling pathways within sperm cells highlights their potential use for the improvement and successful application of assisted reproductive technologies.

Keywords: gasotransmitters; hydrogen sulfide; interaction; metabolism; nitric oxide; spermatozoa

1. Introduction

Since the late 1980s, there has been increasing interest in the role of gaseous molecules in cellular physiology and pathology. Up until 1987, when Palmer et al. [1] identified the endothelium-derived relaxing factor to be nitric oxide (NO), this gas was regarded as a toxic agent. In the same year, Brüne and Ullrich [2] found that carbon monoxide (CO) inhibits platelet aggregation, enhancing guanylyl cyclase (GC) activity. With the discovery of the endogenous production of hydrogen sulfide (H_2S) in rat and human brains [3], the term gasotransmitters emerged to set these three gases apart from the other known types of cellular messengers such as neurotransmitters and humoral factors [4]. Significant advances have been made in the area of gasotransmitters in the vascular [5,6], nervous [7,8], and digestive [9] systems. In contrast to the extensive literature available on NO [10,11], the role of H_2S in male reproduction is less explored and deserves further attention [12]. This review aims to illustrate the role of NO and H_2S in spermatozoa, and also includes recent advances in other cell types that may be potentially relevant to sperm biology. The spermatozoon represents one of the most diverse and specialized cells that originates from the spermatogonial cells in the seminiferous tubules of the testicles. Before leaving the male reproductive tract, the sperm cells undergo epididymal maturation, that is, a series of structural and biochemical changes resulting in the acquisition of fertilization ability and motility [13]. The full fertilization potential is not reached before the sperm cells go through the capacitation within the female reproductive tract. Capacitation involves plasma membrane changes initiated by the loss of cholesterol, also affecting the ion intracellular concentrations and the activity of specific enzymes (e.g., protein kinase A (PKA)). The series of these events results in different movement

patterns, sperm hyperactivation, and finally allows the occurrence of an acrosomal reaction that is the exocytosis of specific enzymes from the sperm head covering vesicle, the acrosome [14].

1.1. NO Metabolism in Spermatozoa

The production of NO in cells is ensured by three isoforms of nitric oxide synthase (NOS) encoded by three different genes [15]. Irrespective of the NOS isoform, the substrates are L-arginine and oxygen (O_2). The first NOS isoform is referred to as neuronal NOS (nNOS; NOS 1), as it was first discovered in neurons and its continuous expression is typical for peripheral and central neuronal cells. Through the action of nitric oxide, nNOS regulates the synaptic activity in the central nervous system and other functions, such as the regulation of blood pressure and smooth muscle relaxation. The second NOS isoform is referred to as inducible NOS (iNOS; NOS 2), since its expression may be induced by cytokines and lipopolysaccharides (LPS) [16]. The iNOS plays an important role in the immune system, as it generates a significant amount of NO, which helps to fight off pathological agents by the fragmentation of their DNA and the inhibition of iron-containing enzymes [17]. The last isoform is the endothelial NOS (eNOS; NOS 3), since it is mostly located in the endothelial cells. The expression of nNOS and eNOS is mainly regulated by Ca^{2+} and calmodulin, which set them apart from iNOS [18], which is activated in the presence of microbial or immunological stimuli [16]. In addition, the NO production by eNOS and nNOS is continuous, but in case of eNOS, it may be enhanced in specific conditions independently of Ca^{2+} signalization. For example, shear stress in the vasculature leads to activation of the phosphoinositide 3-kinase (PI3K) and protein kinase B (Akt) pathways resulting in phosphorylation and activation of eNOS [15]. All three NOS isoforms have been described in the sperm cells of several mammalian species (Table 1) [19]. Interestingly, the pattern of NOS distribution in sperm cells seems to differ across species; for instance, eNOS is localized in the flagellum of human [20], but not bull [21], spermatozoa. Moreover, eNOS is also localized in the equatorial and post-acrosomal regions of morphologically normal human spermatozoa [22]. Aberrant eNOS distribution is often observed in morphologically abnormal spermatozoa and negatively correlates with sperm motility [22]. Furthermore, it is still unclear whether the physiological state (e.g., capacitation) of sperm cells may affect NOS distribution. In a recent study in capacitated boar spermatozoa, Staicu et al. [23] found that the eNOS and nNOS are mainly distributed in the sperm head, whereas iNOS is localized in both the sperm head and the flagellum. The study also suggested a link between NOSs distribution and sperm normal function (capacitation, acrosome reaction, tyrosine phosphorylation, and Ca^{2+} flux). In contrast to boar spermatozoa [23], in epididymal tomcat spermatozoa, all three NOS isoforms are localized in the flagellum and in the cytoplasmic droplet [24]. In murine spermatozoa, the expression of iNOS influences the reproductive outcome [25]. In particular, Yang et al. [25] found that iNOS knockout mice displayed higher fertilization rates, suggesting an iNOS inhibitory effect on sperm fusion with the oocyte. Interestingly, the rate of blastocyst formation was not influenced in any knockout mice. Similarly, the function of pre-ejaculated sperm was unaffected in any NOS knockout [25].

Table 1. The presence and localization of nitric oxide synthases (NOSs) in sperm of different species.

Species	NOS Isoform	Localization	Reference
Man	nNOS eNOS	Head, tail Head	[26] [22]
Mouse	nNOS, iNOS, eNOS	n/a	[27]
Bull	nNOS eNOS	Head, tail Head	[21]
Boar	nNOS iNOS eNOS	Head Head, tail Head	[23]
Stallion	nNOS, eNOS	n/a	[28]
Tomcat	nNOS, iNOS, eNOS	Tail, cytoplasmic droplet	[24]

This table was adapted from Staicu and Matas Parra [19] and modified for the purpose of this review. n/a, not available; NOS, nitric oxide synthase; nNOS, neuronal NOS; iNOS, inducible NOS; eNOS, endothelial NOS.

1.2. H_2S Metabolism in Spermatozoa

The cellular enzymatic production of H_2S is mainly ensured by cystathionine β-synthase (CBS), cystathionine γ-lyase (CSE), and 3-mercaptopyruvate sulfurtransferase (3-MST). Both CBS and CSE are pyridoxal 5′-phosphate-dependent enzymes located in the cytosol, while 3-MST is a zinc-dependent enzyme that is mostly found in the mitochondria [29]. Under stress conditions, CSE can be translocated from the cytosol into the mitochondria, producing H_2S and increasing adenosine triphosphate (ATP) production [30]. Common substrates for H_2S production are L-homocysteine and L-cysteine, which can be obtained by the methionine transulfuration pathway or directly from the diet [31]. The metabolism of α-ketoglutarate (α-KG) represents an alternative source of H_2S [32]. The production of H_2S by 3-MST involves two pathways: a traditional one coupled with cysteine aminotransferase (CAT) and α-KG, and the other one coupled with D-amino acid oxidase (DAO) and D-cysteine [32]. Another pathway for enzymatic production of H_2S may be the reduction of thiols by catalase [33]. Moreover, H_2S can be also oxidized by catalase, so this enzyme seems to play an important role in H_2S metabolism [34]. In addition, mitochondrial complex I is another potentially important source of H_2S due to the high cysteine concentration compared to the one found in the cytosol. Non-enzymatic synthesis arises from persulfides and polysulfides or from the cellular reservoir of bound sulfur and acid-labile sulfur [29]. In regard to bound sulfur, alkaline conditions (pH > 8.4) within neuronal cells promote the release of H_2S in the presence of glutathione (GSH) and cysteine [35]. On the other hand, acid-labile sulfides are not a likely source of H_2S, since their release requires a drop of the pH value to below 5.5 [36]. The catabolism of H_2S is poorly understood [37] and seems to occur mostly within the mitochondria, thanks to enzymes capable of H_2S oxidation: sulfide quinone oxidoreductase (SQR), thiosulfate transferase (TST), and sulfite oxidase [32]. Other enzymes also participate in H_2S catabolism, such as ethylmalonic encephalopathy 1 (ETHE1) protein, which continues the oxidation of sulfide initiated by SQR [38]. Moreover, the enzyme cysteine dioxygenase should be mentioned, as it controls the cellular levels of cysteine, and thus contributes to maintaining low levels of H_2S/sulfane sulfur pools [38]. The non-enzymatic catabolism pathway occurs via interaction of H_2S with O_2, hydrogen peroxide (H_2O_2), superoxide (O_2^-), and peroxynitrite ($ONOO^-$) [32].

There is lack of information regarding the expression and distribution of H_2S-generating enzymes in sperm cells. To the best of the authors' knowledge, only one study has quantified the expression of CBS and CSE in sperm samples [39]. In this study, the authors found that oligoasthenozoospermic and asthenospermic men show reduced levels of H_2S in the seminal plasma compared to fertile men. Interestingly, asthenospermic men show reduced expression of CBS but not CSE. The localization of the H_2S-generating enzymes within the sperm cells is also still unknown.

2. Mechanisms of Action of NO in Spermatozoa

Substantial information is available regarding the role of NO in crucial sperm processes prior to fertilization, such as capacitation, hyperactivation, acrosome reaction, and zona pellucida binding [19,40–42]. Furthermore, the role of NO has been widely investigated during semen handling and storage [43,44]. So far, three main pathways of NO within the sperm cell have been established [19].

The primary target of NO is the soluble guanylyl cyclase (sGC) that serves as the NO receptor. The most common sGC isoform found in cytosolic fractions consists of two subunits: α1 and β1. Each subunit contains four domains: N-terminal heme-NO/O_2 binding (H-NOX), Per/Arnt/Sim domain (PAS), coiled-coil domain (CC; helical d.), and C-terminal catalytic domain [45]. The H-NOX domain of the β1 subunit is the one responsible for the interaction with NO through bounded heme. Upon the binding of NO to the heme group, a cascade of conformational changes of the other domains results in the activation of catalytic activity of the sGC, as demonstrated in vivo using human neuroblastoma-derived cells [46]. The kinetics of the sGC molecule and the interaction with NO was extensively studied by Sürmeli et al. [47] with in vivo implications. The study revealed the relationship between ATP, guanosine-5′-triphosphate (GTP), and NO to the activity of sGC. The ATP binding to the allosteric site (pseudosymmetric to the catalytic domain) gives selectivity of sGC for GTP and affects the enzyme activity at different concentrations of NO [47]. The binding of NO to sGC leads to the production of cyclic guanosine monophosphate (cGMP) [48], which participates in the acrosome reaction of bovine [49] and human [50] spermatozoa. Among cGMP targets, the cyclic nucleotide gated (CNG) channels are one point of interest, since they can be found in the flagellum and affect the Ca^{2+} influx during capacitation of bovine and murine spermatozoa [51,52]. The cGMP also activates cGMP-dependent protein kinase (PKG), an enzyme responsible for phosphorylation of serine/threonine in proteins important for sperm capacitation [19]. Moreover, PKG contributes to the activation of other macroscopic ion currents responsible for maintaining elevated Ca^{2+} levels for longer periods of time during capacitation [52]. An increased production of cGMP also prevents the degradation of cAMP by the phosphodiesterase type 3 (PDE3), as both nucleotides compete for the catalytic site of the enzyme [19]. On the other hand, the intracellular increase of Ca^{2+} may be explained by an extracellular signalization (e.g., progesterone), resulting in sperm-specific Ca^{2+} channel (CatSper) activation and a consequential increase in cGMP production [53].

In addition to the indirect involvement of NO in the cAMP/protein kinase A (PKA) pathway, NO directly acts on adenylyl cyclase (AC) with a dual effect: An activator at small concentrations (murine and human spermatozoa) [54], and an inhibitor at high concentrations (in vitro) [55]. The latter study [55] was performed on transmembrane adenylate cyclase (tmAC), whose function in sperm biology is controversial, despite the fact that all isoforms of tmAC were localized within the cell [56]. In continuation, the tyrosine phosphorylation of proteins is also achieved by the activity of NO on the extracellular signal-regulated kinase (ERK) pathway. NO interacts with the cysteine of Ras proteins, and consequentially several kinases are activated (Raf, MEK, and ERK 1/2) resulting in tyrosine phosphorylation, which contributes to mammalian sperm capacitation [57].

A third mechanism of action occurs at high concentrations of NO, which directly provokes a post-translational modification of proteins, reversibly by S-nitrosylation or irreversibly by tyrosine nitration [40]. Within the human spermatozoa, more than 200 proteins have been identified that are modified by NO via the process of S-nitrosylation [58], which is the covalent union of NO and sulfur of cysteine, forming a nitrosothiol group (-SNO) within the molecule. Moreover, S-nitrosylation is involved in a variety of cellular processes such as energy production, motility, ion channel function, or antioxidative mechanisms [41]. On the other hand, tyrosine nitration is achieved through interaction between NO and $ONOO^-$. Interestingly, the levels of tyrosine nitration and the production of $ONOO^-$ are increased during mammalian sperm capacitation [41].

In mammals, the major source of NO catabolism seems to be the reaction with O_2, forming nitrites [29], or with hemoglobin, forming nitrates [59]. The rapid reaction of NO with thiols [29] and other reactive oxygen species (ROS) represents other possible ways of catabolism [17].

3. Mechanisms of Action of H_2S in Spermatozoa

Regarding the targets of H_2S within the sperm cell, little information is available. Recently, Wang et al. [39] investigated the influence of H_2S on spermatogenetic failure induced by administration of LPS, which lead to the phosphorylation of mitogen-activated protein kinases (MAPKs), a complex of three downstream enzymes (ERK, C-Jun N-terminal kinase, (JNK), and p38) with pro-inflammatory activity. The injection of the synthetic H_2S donor GYY4137 attenuated the effect of LPS by modulating the MAPK pathway and affecting the activity of JNK, ERK, and p38 enzymes. Furthermore, the application of the H_2S donor GYY4137 led to sperm motility improvement in asthenozoospermic men with H_2S deficiency [39]. In boar and mouse semen, Zhao et al. [60] found that Na_2S, a fast H_2S releasing donor, decreases sperm motility by disrupting multiple signaling pathways, which mainly include: decreased ATPase activity, inhibition of Akt, and activation of the adenosine 5′-monophosphate-activated protein kinase (AMPK) and phosphatase and tensin homologue (PTEN) pathways. The AMPK pathway affects spermatogenesis and performs a crucial role in sperm metabolism and the motility of various mammalian species (e.g., rats, stallions, humans) [61]. On the other hand, the activation of the PI3K/Akt pathway can help to counteract the effects of oxidative stress. Xia et al. [62] observed the activation of the PI3K/Akt pathway in varicocelized (VC) mice after administration of the H_2S donor (GYY4137) compared to the VC group. The phosphorylation of PI3K p85 and Akt positively correlated with sperm motility, decreased oxidative stress, and reduced epididymal cell apoptosis [62].

However, more potential targets for H_2S may be expected. H_2S is known to interact with proteins during post-translational modification [63]. The interaction of H_2S with cysteine results in the conversion of cysteine -SH groups to -SSH, and the term S-sulfhydration is used to describe this kind of protein modification [64]. Moreover, Mustafa et al. [64], upon the observation of H_2S interaction with glyceraldehyde 3-phosphate dehydrogenase (GAPDH), suggested H_2S to be antagonistic to NO, since H_2S tends to increase cysteine reactivity rather than decrease it, as in the case of NO. This finding may have interesting implications in sperm biology, since GAPDH is a glycolytic enzyme involved in sperm motility [65,66]. In addition, a sperm-specific isoform (GAPDS) with constitutional differences and more specific function is found within sperm cells [67]. The GAPDS is expressed only in male germ cells and performs a narrower range of tasks compared to the somatic isoform (GAPDH). Doubtlessly, the main task is to ensure energy for sperm motion. As a result, the knockout of the gene encoding GAPDS results in a significant motility decrease, while the O_2 consumption by mitochondria and the ATP production by oxidative phosphorylation (OXPHOS) are maintained [68].

Recently, the term S-sulfhydration has been substituted by a more accurate one, namely persulfidation, as no hydration occurs during the reaction of H_2S and cysteine -SH group [69,70]. This raises more questions about the direct involvement of H_2S in cellular signaling, as the sulfur atoms of cysteine and H_2S are reduced to -2 oxidation state and oxidation to S^- is required before persulfidation can occur [70,71]. The slow rate of H_2S autooxidation, the lower reactivity of H_2S compared to persulfides/polysulfides, and the low specificity imply that oxidized products of H_2S (i.e., polysulfides and persulfides) are the actual cellular messengers [70,72]. Mishanina et al. [72] proposed that enzymes producing persulfides, such as sulfurtransferases (e.g., 3-MST, rhodanese), CSE, CBS or SQR, transfer persulfides to another protein directly or via a secondary carrier, which would create targeting specificity. Thus, the persulfide transfer (transpersulfidation) would be the most likely mechanism of signalization of H_2S.

4. The Role of NO and H_2S in Oxidative Stress

The presence of NO and H_2S within semen may be linked to physiological processes or pathological states depending on the concentration (Table 2). Whereas at low concentrations ROS play a key role in sperm function (e.g., capacitation, acrosome reaction), above physiological levels they provoke oxidative stress and sperm damage [73,74]. Apart from ROS, reactive nitrogen species (RNS) [75] and reactive sulfur species (RSS) [76] are also involved in several cellular processes. To maintain the balance between physiological signal transduction and over-accumulation of reactive species, antioxidants such

as super oxide dismutase (SOD), catalase, or the glutathione peroxidase (GPX)/glutathione reductase (GR) system are present within the seminal plasma [77]. Moreover, the sperm cell itself has an intrinsic antioxidant system, involving antioxidants such as peroxiredoxins and thioredoxins, in addition to the above-mentioned seminal plasma antioxidants [78]. Nevertheless, it should be emphasized that sperm cells possess limited antioxidant capacity due to the low content of cytoplasm and the high content of polyunsaturated fatty acids (PUFA), which make the male gamete vulnerable to oxidative stress [73]. A study by Moretti et al. [79] demonstrates that the increased ROS production in infertile men leads to impairment of sperm parameters (e.g., motility and viability) and alteration of the antioxidant system within the cell. As mitochondria are the main source of ROS within the spermatozoon, as well as a major source of energy for movement, the decrease in sperm motility in response to oxidative stress may be linked to alterations of mitochondrial activity [80].

Table 2. The effects of nitric oxide (NO) and hydrogen sulfide (H_2S) on cellular function.

PHYSIOLOGICAL CONCENTRATION		SUPRAPHYSIOLOGICAL CONCENTRATION	
NO	**H_2S**	**NO**	**H_2S**
↓ lipid peroxidation*	**ROS scavenging activity***	**↑ lipid peroxidation**	**↓sperm motility**
	↑ antioxidant capacity	**↑ DNA damage**	**↑ ROS levels**
	○ ↑SOD activity	**↑ protein damage**	**↓ mitochondrial activity**
	↑ mitochondrial activity	**↑ apoptosis***	○ Complex IV inhibition*
	↑ sperm motility	○ membrane hyperpolarization*	
	↑ DNA integrity	○ cytochrome C release*	
	apoptosis prevention	**↓ mitochondrial activity**	
	○ ↑ HSP 70 expression	○ complex IV inhibition*	
	○ ↓ Caspase 3 expression	**↑ $ONOO^-$ generation**	
	○ Bax/Bcl-2 ratio preservation	○ mitochondrial activity inhibition	
	Cryoprotection	▪ complexes I and II inhibition*	
	○ ↑ HSP 70 expression	▪ Mn-SOD inactivation*	
	▪ ↑ sperm motility	▪ Succinate dehydrogenase inactivation*	
	▪ ↑ membrane integrity	○ ↓ glycolysis	
	▪ ↑ DNA integrity	○ ↑ thiol oxidation	
	▪ ↓ % abnormal sperm		

* Effects seen in other systems rather than just the male reproductive system. Bax, Bcl-2-associated X protein; Bcl-2, B-cell lymphoma 2 protein; HSP, heat-shock protein; ROS, reactive oxygen species; SOD, superoxide dismutase; $ONOO^-$, peroxynitrite. While bold letter indicates topic within the table, circles and squares indicates 1st and 2nd level subtopics.

4.1. NO and Reactive Nitrogen Species

NO is a free radical representing the main source of RNS, which originate from the interaction of NO with O_2 and O_2^- to produce nitrogen dioxide (NO_2), dinitrogen trioxide (N_2O_3), dinitrogen tetraoxide (N_2O_4), $ONOO^-$, and nitroxyl (HNO) [40]. Ultimately, excessive RNS can be responsible for lipid, protein, and DNA impairment [81]. NO is the least reactive radical often connected with PUFA peroxidation. As a free radical, increased concentrations of NO within the sperm cell are associated with male infertility [79]. In this way, aminoguanidine, an NOS inhibitor, protects the sperm cells against the detrimental consequences of oxidative stress both in vivo and in vitro [82,83]. Yet, it should be mentioned that NO may also stop radical chain propagation through interaction with the lipid peroxyl radical (an intermediate of lipid peroxidation) to form oxidized forms of nitrosated fatty acid species [84]. Apart from its physiological role, NO pathological accumulation at µM concentrations in mitochondria inhibits cellular respiration, while at mM concentrations it may also lead to membrane hyperpolarization, cytochrome c release, and apoptosis [10]. The inhibition of mitochondrial respiratory activity by NO itself is done through reversible inhibition of complex IV upon the binding of NO to the heme group of cytochrome oxidase [84]. In addition to NO, the free radical $ONOO^-$ is one of the most potent RNS involved in various signaling pathways, and has potential pathological effects when left uncontrolled by the antioxidant cellular defense [85]. The overproduction of $ONOO^-$ leads

to the inhibition of mitochondrial activity through the inactivation of electron transport complexes I (NADH dehydrogenase) and II (succinate dehydrogenase). The function of SOD can be also affected by $ONOO^-$ through tyrosine nitration [84]. Various types of SOD are known, of which two types are found in eukaryotic organisms: Mn-SOD located in the mitochondria and Cu/Zn-SOD mostly located in the cytosol [86]. Mn-SOD is inactivated by $ONOO^-$ [84]. The influence of $ONOO^-$ overproduction on human spermatozoa was investigated by Uribe et al. [87], revealing a decrease in the mitochondrial membrane potential and motility. These observations led to the hypothesis that decreased ATP production could be behind the observed effects. This hypothesis was later confirmed by the same research group [88], as the application of peroxynitrite interfered with ATP production via OXPHOS, and also via glycolysis. Moreover, thiol oxidation, resulting from the reaction of $ONOO^-$ with sulfhydryl groups of cysteine, was related to decreased sperm motility. The process affected both the sperm head and the principal piece, and as a possible explication of motility loss, a thiol oxidation of the sperm axoneme was suggested [89]. In addition, Uribe et al. [90] observed mitochondrial permeability transition (MPT) under nitrosative stress with biochemical traits of MPT-driven necrosis. On the contrary, Serrano et al. [91] found that, although peroxinitrite induces oxidative stress in boar sperm, leading to lipid peroxidation and motility loss, it does not affect mitochondrial membrane potential.

4.2. H_2S and Reactive Sulfur Species

The most recent and complex definition describes the RSS as those molecules which contain at least one redox-active sulfur atom or sulfur-containing functional group in their structure, and are capable of either oxidizing or reducing biomolecules under physiological conditions to trigger or propagate a noticeable cellular signal or wider biological event [92]. The need for this new definition comes from the extensive research done in the area of cellular signaling involving RSS. Mishanina et al. [72] list a wide range of biologically active RSS with H_2S as a common precursor. Like RNS and ROS, the concentration of RSS is crucial for physiological activity, as in supraphysiological concentrations, RSS exert a negative effect on sperm cells [40]. In a study by Wang et al. [39], asthenozoospermic men showed decreased H_2S concentrations in seminal plasma, and the application of a H_2S donor (GYY4137) improved the total and progressive sperm motility. In the same study, the negative effect on human sperm motility and hypermotility was seen after 5 μM NaHS treatment, which probably caused the fast release of H_2S in a supraphysiological concentration. Similarly, Zhao et al. [60] reported that the administration of Na_2S, both in vitro (25–100 μM) and in vivo (10 mg/kg of body weight), led to negative effect on boar and mouse sperm motility, respectively. The observed negative effects of H_2S donors may be due to the inhibition of ATP production. Particularly, the inhibition of mitochondrial complex IV takes place when using an NaHS donor in concentrations exceeding 10 μM in various cell lines [93]. Finally, high concentrations of a H_2S donor (50 μM Na_2S) promote oxidative stress, measured as the concentration of H_2O_2, in boar sperm samples [60].

4.3. H_2S Antioxidant Properties

Great focus has been dedicated to the antioxidant properties of H_2S as a reducing agent (Table 2) [94]. At low concentrations H_2S and its dissociated form, (HS^-), can directly scavenge ROS and RNS (e.g., O_2^-, H_2O_2 or peroxynitrates) [35]. Bearing in mind the very low H_2S cellular concentration (sub-micromolar), the direct scavenging activity seems to be of lesser importance compared to other antioxidants (e.g., GSH) [35,95]. On the other hand, indirect augmentation of antioxidant capacity has been documented in several studies. In a study by Li et al. [96], the application of NaHS as a H_2S donor led to increased SOD activity and decreased ROS levels in testicular germ cells exposed to heat stress. Moreover, mitochondrial dysfunction characterized by increased ATP depletion, O_2 consumption, and ROS generation was also reduced after NaHS application. The results also indicated that H_2S may prevent cellular apoptosis. In a similar study, Ning et al. [97] used another H_2S donor, GYY4137, to test its effect on heat-induced damage in testicular cells. In agreement with Li et al. [96], H_2S donor administration led to increased SOD expression and reduced the number of apoptotic cells. The

authors also measured the expression of several proteins of the mitochondrial apoptotic pathway: Bax, Bcl-2, and caspase 3. The application of GYY4137 reduced the expression of Bax in heat-exposed testicular cells and preserved the expression of Bcl-2 compared to the group without treatment [97]. The ratio between Bax (pro-apoptotic factor) and Bcl-2 (anti-apoptotic factor) protein is crucial in apoptosis activation [98]. As a consequence, the authors also found that the expression of caspase 3 was also reduced in the GYY4137-treated group [97]. Caspase 3 is a signaling enzyme of various pathways, whose activation leads to inevitable apoptosis [99]. The effects observed by Ning et al. [97] are attributed to the increased expression of heat shock protein 70 (HSP 70) after GYY4137 application. The expression of HSP 70 helps to prevent cell apoptosis during temperature-induced stress conditions in testicular cells [100], preserves sperm motility in cryopreserved bull spermatozoa [101], and protects proteins and DNA under stress conditions [102]. Using antioxidant sericin, the increased expression of HSP 70 led to improved semen quality after cryopreservation [103].

5. NO and H_2S Interactions

There is growing evidence indicating that H_2S and NO share common targets and interact with each other [104]. Most information about the interactions of H_2S and NO come from research on the cardiovascular system. The studies dedicated to this topic demonstrate the interaction on several levels: shared signaling targets (Figure 1), metabolic regulation of each other, and interaction between metabolites of both gasotransmitters (Figure 2) [59]. For example, the interaction of H_2S and NO leads to the formation of polysulfides, which are more reactive than H_2S, and thus seem to be novel RSS signal conductors [70].

With respect to the common signaling targets for H_2S and NO, the MAPK pathway is one point of interest. The MAPK pathway includes four main cascades, namely, ERK 1/2, JNK, p38, and ERK 5, and it is known to participate in sperm capacitation, motility, and acrosome reaction [105]. While H_2S decreases phosphorylation by MAPK in the testis [39], NO activates MAPK participating in the tight-junction dynamics of Sertoli cells [106]. This MAPK regulation by H_2S and NO may also be of interest regarding sperm cells, as it is a crucial pathway affecting sperm motility, morphology, and capacitation [57,107]. For the first time in human spermatozoa, Silva et al. [107] identified JNK, which represent a subfamily of MAPKs also referred to as stress-activated protein kinases (SAPKs), as they are activated by phosphorylation under stress conditions (e.g., oxidative stress). The same authors observed a negative correlation of JNK phosphorylated levels with total and progressive motility. Furthermore, the application of NaHS in mice decreased the activity of MAPKs in the blood–testis barrier of samples exposed to oxidative stress induced by LPS [39]. Thus, it seems that the phosphorylation of MAPKs is attenuated by H_2S. On the other hand, exposure of cells to peroxynitrate activates all three of the major subfamilies of MAPKs (p38, JNK, ERK) in rat liver epithelial cells [108]. Yet, the effect of the two gasotransmitters on the MAPK pathway within a sperm cell remains to be investigated. Other common targets in somatic cells for both gasotransmitters are the Ca^{2+} channels [29,35] and K^+ channels [29,35,59]. The regulation of Ca^{2+} currents in sperm is of particular interest, as CatSper are involved not only in sperm capacitation [109], but also in sperm hyperactivation, acrosome reaction, and chemotaxis [53,110]. The hypothesis of NO involvement in chemotaxis through affection of the ion channel function may seem intriguing, as Miraglia et al. [111] observed a positive influence of NO on sperm migration. On the other hand, a recent study by Wilińsky et al. [112] showed only temporal negative influence of H_2S on sperm chemotaxis, probably due to motility inhibition. The specific mechanism and extent of involvement of the CatSper channels in the previously mentioned processes is still a matter of debate [113,114]. In addition, the opening of K^+ channels induces membrane hyperpolarization, representing the predominant process during capacitation. The regulation of K^+ channels also affects ATP generation by mitochondria, and thus the activation promotes progressive sperm movement, together with hyperactivity [115].

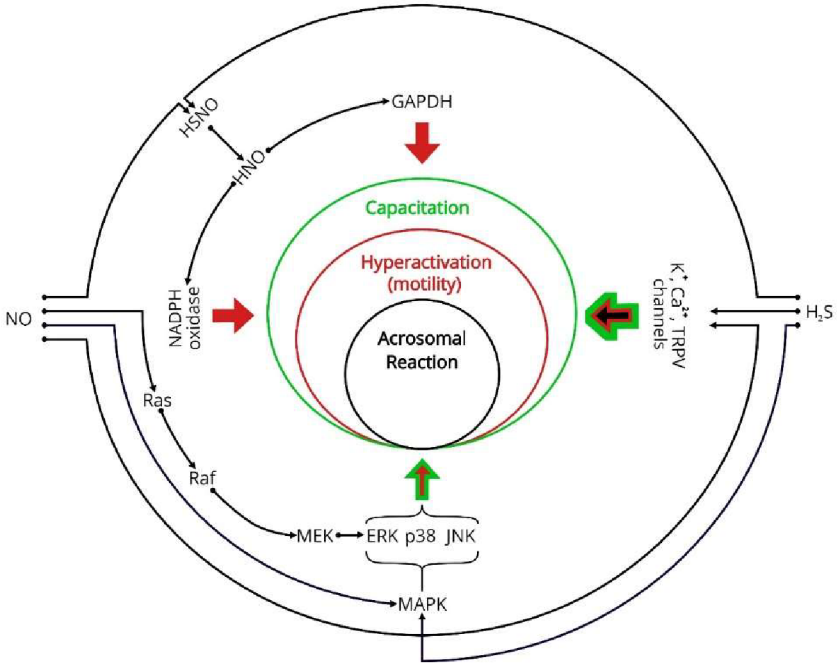


Figure 1. Common targets of nitric oxide (NO) and hydrogen sulfide (H_2S). The scheme displays the cohesion of H_2S and NO common targets within a cell, focusing on the most sperm-relevant enzymes and proteins. The function of NADPH oxidase and GAPDH directly affects sperm motility, as the latter requires ATP production. The sperm ion channels affect not only sperm function (capacitation, hyperactivation, acrosomal reaction), but also the outcome of the fertilization process. The MAPK complex influences the capacitation and hyperactivation of sperm cells. Colors of arrows indicate the relation with sperm biological process marked by the corresponding color. ERK, extracellular signal-regulated kinase; GAPDH, 3-phosphate dehydrogenase; HNO, nitroxyl; HSNO, thionitrous acid; H_2S, hydrogen sulfide; JNK, C-Jun N-terminal kinase; MAPK, mitogen-activated protein kinases; MEK, MAPK/ERK kinase; NADPH, nicotinamide adenine dinucleotide phosphate; NO, nitric oxide; Raf, rapidly accelerated fibrosarcoma kinase; TRPV, transient receptor potential vanilloid.

Attention should also be given to the transient receptor potential (TRP) channels, which affect male fertility potential, starting from spermatogenesis, through sperm maturation, to sperm function. The TRP channels are involved in sperm thermotaxis, forming a group of 30 Ca^{2+} ion channels, which can be divided into seven families [116,117]. Some channels of the subfamily of TRP vanilloid (TRPV) can be activated by H_2S [29] and NO through S-nitrosylation [118]. The ion channel TRPV type 4 (TRPV4) was very recently demonstrated to participate in human sperm capacitation and hyperactivation [119]. The TRPV4 channel function is temperature dependent and is probably modulated by tyrosine phosphorylation [119]. Following the authors' model, TRPV4 mediates Na^+ influx and the consequential membrane depolarization necessary for activation of other crucial capacitation-related ion channels (e.g., CatSper). The authors immunolocalized TRPV4 in the flagellum and acrosome of human spermatozoa. Another TRP channel (TRPV1) was immunolocalized by Kumar et al. [120] in the acrosome and in the flagellum of bull spermatozoa. The authors observed a correlation of TRPV1 with progressive sperm motility, hyperactivity, capacitation, and acrosome reaction. TRPV1 was also observed to play an important role in the capacitation of boar spermatozoa [121]. The activation of TRPV1 leads to membrane depolarization through Na^+ influx and the consequential activation of voltage-gated Ca^{2+} channels. The same effect was also observed in mouse spermatozoa [122]. In a study by Bernabò et al. [121], the TRPV1 localization displayed two patterns in ejaculated spermatozoa: in the majority of spermatozoa, TRPV1 was found in the post-acrosomal region, while around 20% of spermatozoa had TRPV1 distributed over the acrosome and in the proximal segment of the midpiece. The authors observed a dramatic shift of this distribution pattern after capacitation, describing the

relocation of TRPV1 to the acrosome and midpiece. Yet the regulation of the TRPV channel by H_2S and NO in spermatozoa of different species remains to be investigated.

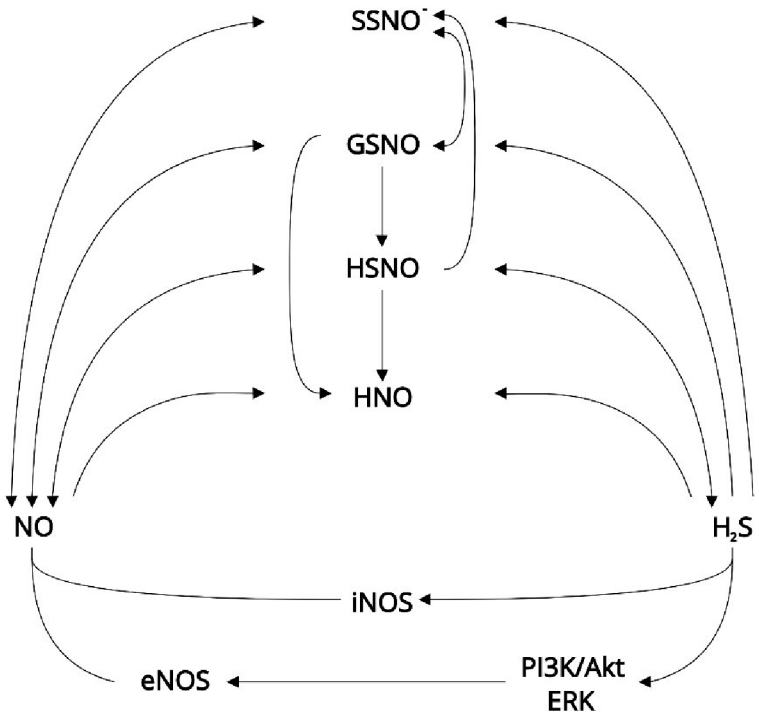


Figure 2. A brief insight into the interactions between NO and H_2S that might be relevant for sperm biology. Akt, protein kinase B; eNOS, endothelial nitric oxide synthase; ERK, extracellular signal-regulated kinase; GSNO, S-nitrosoglutathione; HNO, nitroxyl; HSNO, thionitrous acid; H_2S, hydrogen sulfide; iNOS, inducible nitric oxide synthase; NO, nitric oxide; PI3K, phosphoinositide 3-kinase. [32,50,116–119,123–130].

A regulatory effect of H_2S on NO production may result from the ability of H_2S to activate the PI3K/Akt and ERK pathways [60]. Using various H_2S donors in CSE knockout mice, H_2S activates eNOS in myocardial cells [123]. The enzymes ERK 1/2 were reported to enhance eNOS sensitivity to Ca^{2+} stimulation in the endothelial cells of the uterine artery [124]. The release of NO upon the activation of MEK/ERK1/2 and PI3K/Akt-dependent eNOS serine 1179 phosphorylation was also observed after H_2O_2 application [125], which describes a cellular mechanism of adaptation to oxidative stress. In contrast, the application of the H_2S donors NaHS and diallyl trisulfide leads to the inhibition of iNOS during inflammation [126]. However, the effect of the interaction between NOS and H_2S is still unclear, indifferent of cell type [127].

A direct interaction between H_2S and NO radicals and their metabolites (e.g., nitrate, nitrite, peroxinitrates) results in the formation of potentially important signaling molecules such as nitrosothiols, thionitrous acid (HSNO), or nitroxyl (HNO) [59,128]. The interaction between NO and H_2S is currently being intensively investigated, as it represents a very complex topic of great physiological importance and results in a plethora of possible outcomes [129]. For instance, HS^- reacts with $ONOO^-$, forming HSNO [59], which seems to be another important source of NO and HNO [130]. In addition, the reaction of HS^- with S-nitrosothiol (SNO) and S-nitrosoglutathione (GSNO) generates several other metabolites (e.g., sulfinyl nitrite ($HSNO_2$) and HSNO) [127]. Within the cardiovascular system, the role of HNO in cellular physiology has received considerable attention [59], with possible interesting implications for sperm cells. Using a HNO donor (Angeli's salt), Andrews et al. [131] demonstrated for the first time that it acts through the sGC/cGMP pathway. HNO also protects PUFA from peroxidation due to its antioxidant properties [132]. The protective ability of HNO should also be considered in the

case of the sperm plasma membrane, as it contains a high amount of PUFA [73]. On the other hand, HNO can increase intracellular levels of H_2O_2 by inhibiting its degradation, and it also reacts with thiol proteins, such as GAPDH, decreasing its activity [132,133]. Sperm-specific GAPDH (GAPDS) is particularly important in sperm cell energetic metabolism [68]. It has been proposed that interaction of NO with H_2S may result in GSNO formation [37]. Yet, the reaction of nitrous acid (HNO_2) with GSH seems to be the most relevant in physiological conditions, compared to the reaction of GSH and NO, which represents another alternative for in vivo GSNO formation [134]. It seems that GSNO serves as an intracellular storage for NO, which can be released by the reaction with GPX or thioredoxin reductase [59]. GSNO can also release stored NO upon reaction with H_2S or HS^- [135], and can also lead to formation of polysulfane species [136]. In addition, Berenyiova et al. [137] proposed that sulfide reaction with GSNO may lead to HNO synthesis. Although HNO was observed to inhibit nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (Nox 2) in the vascular system [59], the form and role of NADPH oxidase in spermatozoa is unclear [11]. Only the isoform Nox 5 has been found in the testis [11] and in human spermatozoa, where it was localized in the flagellum, midpiece, and acrosome and was positively associated with motility [138]. Recently, nitrosopersulfide ($SSNO^-$) was suggested as a more probable, effective, resistant, and specific NO donor than GSNO [139,140]. It was also suggested that $SSNO^-$ is formed in the presence of excessive sulfide, in addition to the other ways of formation [139]. On the other hand, Wedmann et al. [141] proposed that under in vivo physiological conditions, $HSNO/SNO^-$ is the most probable signaling molecule (via trans-nitrosation), which can also cause HNO formation.

6. Conclusions

In conclusion, the roles of H_2S and NO in sperm cells still leave many unanswered questions. Surprisingly, even after two decades of intensive investigation, the exact mechanism of action of H_2S is still unclear. The delicately tuned relationship and wide range of molecular targets of these two gasotransmitters within the cell highlight the necessity for further research. Growing evidence indicates that the research on the male gamete should not only take into account the sole action of each gasotransmitter, but it should also focus on investigating the interaction between NO and H_2S.

Author Contributions: Conceptualization, M.K., J.L.R.-S. and E.P.; writing—original draft preparation, M.K.; writing—review and editing, J.L.R.-S and E.P. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

3-MST	3-mercaptopyruvate sulfurtransferase
AC	adenylyl cyclase
Akt	protein kinase B
AMPK	adenosine 5′-monophosphate–activated protein
ATP	adenosine triphosphate
Bax	Bcl-2-associated X protein
Bcl-2	B-cell lymphoma 2 protein
CAT	cysteine aminotransferase
CatSper	sperm specific Ca^{2+} channels
CBS	cystathionine β-synthase
CC	coiled-coil domain
cGMP	cyclic guanosine monophosphate
CNG	cyclic nucleotide gated (channels)
CSE	cystathionine γ-lyase
DAO	D-amino acid oxidase

eNOS	endothelial nitric oxide synthase
ERK	extracellular signal-regulated kinase
ETHE1	ethylmalonic encephalopathy 1 protein
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GAPDS	sperm-specific glyceraldehyde 3-phosphate dehydrogenase
GPX	glutathione peroxidase
GR	glutathione reductase
GSH	glutathione
GTP	guanosine-5′-triphosphate
H-NOX	N-terminal heme-NO/O_2 binding (domain)
HSP	heat shock protein
iNOS	inducible nitric oxide synthase
JNK	C-Jun N-terminal kinase
MAPK	mitogen-activated protein kinases
MEK	MAPK/ERK kinase
MPT	mitochondrial permeability transition
NADH	nicotinamide adenine dinucleotide
NADPH	nicotinamide adenine dinucleotide phosphate
nNOS	neuronal nitric oxide synthase
NOS	nitric oxide synthase
Nox_2	nicotinamide adenine dinucleotide phosphate oxidase 2
OXPHOS	oxidative phosphorylation
PAS	Per/Arnt/Sim (domain)
PDE3	phosphodiesterase type 3
PI3K	phosphoinositide 3-kinase
PKG	cGMP-dependent protein kinase
PTEN	phosphatase and tensin homologue
PUFA	polyunsaturated fatty acids
Raf	rapidly accelerated fibrosarcoma kinase
RNS	reactive nitrogen species
ROS	reactive oxygen species
RSS	reactive sulfur species
SAPK	stress-activated protein kinases
sGC	soluble guanylyl cyclase
SQR	sulfide quinone oxidoreductase
TRP	transient receptor potential (channels)
TRPV	TRP vanilloid (channels)
TST	thiosulfate transferase
VC	varicocelized
α-KG	α-ketoglutarate

References

1. Palmer, R.M.J.; Ferrige, A.G.; Moncada, S. Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. *Nature* **1987**, *327*, 524–526. [CrossRef]
2. Brune, B.; Ullrich, V. Inhibition of platelet aggregation by carbon monoxide is mediated by activation of guanylate cyclase. *Mol. Pharmacol.* **1987**, *32*, 497–504.
3. Goodwin, L.R.; Francom, D.; Dieken, F.P.; Taylor, J.D.; Warenycia, M.W.; Reiffenstein, R.J.; Dowling, G. Determination of sulfide in brain tissue by gas dialysis/ion chromatography: Postmortem studies and two case reports. *J. Anal. Toxicol.* **1989**, *13*, 105–109. [CrossRef] [PubMed]
4. Wang, R. Two's company, three's a crowd: Can H_2S be the third endogenous gaseous transmitter? *Faseb J.* **2002**, *16*, 1792–1798. [CrossRef] [PubMed]
5. Cirino, G.; Vellecco, V.; Bucci, M. Nitric oxide and hydrogen sulfide: The gasotransmitter paradigm of the vascular system. *Br. J. Pharmacol.* **2017**, *174*, 4021–4031. [CrossRef] [PubMed]

6. Holwerda, K.M.; Karumanchi, S.A.; Lely, A.T. Hydrogen sulfide: Role in vascular physiology and pathology. *Curr. Opin. Nephrol. Hypertens.* **2015**, *24*, 170–176. [CrossRef]
7. Panthi, S.; Manandhar, S.; Gautam, K. Hydrogen sulfide, nitric oxide, and neurodegenerative disorders. *Transl. Neurodegener.* **2018**, *7*, 3. [CrossRef] [PubMed]
8. Paul, B.D.; Snyder, S.H. Gasotransmitter hydrogen sulfide signaling in neuronal health and disease. *Biochem. Pharmacol.* **2018**, *149*, 101–109. [CrossRef] [PubMed]
9. Wallace, J.L.; Ianaro, A.; de Nucci, G. Gaseous Mediators in Gastrointestinal Mucosal Defense and Injury. *Dig. Dis. Sci.* **2017**, *62*, 2223–2230. [CrossRef]
10. Buzadzic, B.; Vucetic, M.; Jankovic, A.; Stancic, A.; Korac, A.; Korac, B.; Otasevic, V. New insights into male (in) fertility: The importance of NO. *Br. J. Pharmacol.* **2015**, *172*, 1455–1467. [CrossRef]
11. Toor, J.S.; Sikka, S.C. Human spermatozoa and interactions with oxidative stress. In *Oxidants, Antioxidants and Impact of the Oxidative Status in Male Reproduction*; Henkel, R., Samanta, L., Agarwal, A., Eds.; Elsevier Inc.: Amsterdam, the Netherlands, 2019; Chapter 1.6; pp. 45–53.
12. Di Villa Bianca, R.D.E.; Sorrentino, R.; Maffia, P.; Mirone, V.; Imbimbo, C.; Fusco, F.; De Palma, R.; Ignarro, L.J.; Cirino, G. Hydrogen sulfide as a mediator of human corpus cavernosum smooth-muscle relaxation. *Proc. Natl. Acad. Sci. USA* **2009**, *106*, 4513–4518. [CrossRef] [PubMed]
13. Sullivan, R.; Mieusset, R. The human epididymis: Its function in sperm maturation. *Hum. Reprod. Update* **2016**, *22*, 574–587. [CrossRef] [PubMed]
14. Stival, C.; Puga Molina, L.C.; Paudel, B.; Buffone, M.G.; Visconti, P.E.; Krapf, D. Sperm capacitation and acrosome reaction in mammalian sperm. In *Sperm Acrosome Biogenesis and Function during Fertilization. Advances in Anatomy, Embryology and Cell Biology*; Buffone, M., Ed.; Springer: Cham, Switzerland, 2016; Volume 220, pp. 93–106.
15. Zhao, Y.; Vanhoutte, P.M.; Leung, S.W.S. Vascular nitric oxide: Beyond eNOS. *J. Pharmacol. Sci.* **2015**, *129*, 83–94. [CrossRef] [PubMed]
16. Lind, M.; Hayes, A.; Caprnda, M.; Petrovic, D.; Rodrigo, L.; Kruzliak, P.; Zulli, A. Inducible nitric oxide synthase: Good or bad? *Biomed. Pharmacother.* **2017**, *93*, 370–375. [CrossRef] [PubMed]
17. Lundberg, J.O.; Gladwin, M.T.; Weitzberg, E. Strategies to increase nitric oxide signalling in cardiovascular disease. *Nat. Rev. Drug Discov.* **2015**, *14*, 623–641. [CrossRef] [PubMed]
18. Förstermann, U.; Kleinert, H. Nitric oxide synthase: Expression and expressional control of the three isoforms. *Naunyn. Schmiedebergs. Arch. Pharmacol.* **1995**, *352*, 351–364. [CrossRef] [PubMed]
19. Staicu, F.D.; Matas Parra, C. Nitric oxide: Key features in spermatozoa. In *Nitric Oxide Synthase—Simple Enzyme-Complex Roles*; Saravi, S.S.S., Ed.; IntechOpen: London, UK, 2017; Chapter 8; pp. 138–154.
20. Lewis, S.E.; Donnelly, E.T.; Sterling, E.S.; Kennedy, M.S.; 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*, 873–878. [CrossRef]
21. Meiser, H.; Schulz, R. Detection and localization of two constitutive NOS isoforms in bull spermatozoa. *Anat. Histol. Embryol.* **2003**, *32*, 321–325. [CrossRef]
22. O'Bryan, M.K.; Zini, A.; Cheng, C.Y.; Schlegel, P.N. Human sperm endothelial nitric oxide synthase expression: Correlation with sperm motility. *Fertil. Steril.* **1998**, *70*, 1143–1147. [CrossRef]
23. Staicu, F.D.; Lopez-Úbeda, R.; Romero-Aguirregomezcorta, J.; Martínez-Soto, J.C.; Matás Parra, C. Regulation of boar sperm functionality by the nitric oxide synthase/nitric oxide system. *J. Assist. Reprod. Genet.* **2019**, *36*, 1721–1736. [CrossRef]
24. 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. [CrossRef] [PubMed]
25. Yang, J.Z.; Ajonuma, L.C.; Rowlands, D.K.; Tsang, L.L.; Ho, L.S.; Lam, S.Y.; Chen, W.Y.; Zhou, C.X.; Chung, Y.W.; Cho, C.Y.; et al. The role of inducible nitric oxide synthase in gamete interaction and fertilization: A comparative study on knockout mice of three NOS isoforms. *Cell Biol. Int.* **2005**, *29*, 785–791. [CrossRef] [PubMed]
26. Herrero, M.B.; Perez, M.S.; Viggiano, J.M.; Polak, J.M.; De Gimeno, M.F. Localization by indirect immunofluorescence of nitric oxide synthase in mouse and human spermatozoa. *Reprod. Fertil. Dev.* **1996**, *8*, 931–934. [CrossRef] [PubMed]

27. Herrero, M.B.; Goin, J.C.; Boquet, M.; Canteros, M.G.; Franchi, A.M.; Perez Martinez, S.; Polak, J.M.; Viggiano, J.M.; Gimeno, M.A.F. The nitric oxide synthase of mouse spermatozoa. *FEBS Lett.* **1997**, *411*, 39–42. [CrossRef]
28. Ortega Ferrusola, C.; Gonzalez Fernandez, L.; Macias Garcia, B.; Salazar-Sandoval, C.; Morillo Rodriguez, A.; Rodríguez Martinez, H.; Tapia, J.A.; Pena, F.J. Effect of cryopreservation on nitric oxide production by stallion spermatozoa. *Biol. Reprod.* **2009**, *81*, 1106–1111. [CrossRef] [PubMed]
29. Kolluru, G.K.; Shen, X.; Yuan, S.; Kevil, C.G. Gasotransmitter heterocellular signaling. *Antioxid. Redox Signal.* **2017**, *26*, 936–960. [CrossRef]
30. Fu, M.; Zhang, W.; Wu, L.; Yang, G.; Li, H.; Wang, R. Hydrogen sulfide (H_2S) metabolism in mitochondria and its regulatory role in energy production. *Proc. Natl. Acad. Sci. USA* **2012**, *109*, 2943–2948. [CrossRef]
31. Olson, K.R.; Straub, K.D. The role of hydrogen sulfide in evolution and the evolution of hydrogen sulfide in metabolism and signaling. *Physiology* **2016**, *31*, 60–72. [CrossRef]
32. Olson, K.R. H_2S and polysulfide metabolism: Conventional and unconventional pathways. *Biochem. Pharmacol.* **2018**, *149*, 77–90. [CrossRef]
33. Olson, K.R.; Gao, Y.; DeLeon, E.R.; Arif, M.; Arif, F.; Arora, N.; Straub, K.D. Catalase as a sulfide-sulfur oxido-reductase: An ancient (and modern?) regulator of reactive sulfur species (RSS). *Redox Biol.* **2017**, *12*, 325–339. [CrossRef]
34. Olson, K.R. Hydrogen sulfide, reactive sulfur species and coping with reactive oxygen species. *Free Radic. Biol. Med.* **2019**, *140*, 74–83. [CrossRef] [PubMed]
35. Shefa, U.; Kim, M.S.; Jeong, N.Y.; Jung, J. Antioxidant and cell-signaling functions of hydrogen sulfide in the central nervous system. *Oxid. Med. Cell. Longev.* **2018**, *2018*, 1873962. [CrossRef] [PubMed]
36. Ishigami, M.; Hiraki, K.; Umemura, K.; Ogasawara, Y.; Ishii, K.; Kimura, H. A source of hydrogen sulfide and a mechanism of its release in the brain. *Antioxid. Redox Signal.* **2009**, *11*, 205–214. [CrossRef] [PubMed]
37. Kimura, H. Hydrogen sulfide and polysulfides as biological mediators. *Molecules* **2014**, *19*, 16146–16157. [CrossRef]
38. Rose, P.; Moore, P.K.; Zhu, Y.Z. H_2S biosynthesis and catabolism: New insights from molecular studies. *Cell. Mol. Life Sci.* **2017**, *74*, 1391–1412. [CrossRef]
39. Wang, J.; Wang, W.; Li, S.; Han, Y.; Zhang, P.; Meng, G.; Xiao, Y.; Xie, L.; Wang, X.; Sha, J.; et al. Hydrogen sulfide as a potential target in preventing spermatogenic failure and testicular dysfunction. *Antioxid. Redox Signal.* **2018**, *28*, 1447–1462. [CrossRef]
40. Otasevic, V.; Stancic, A.; Korac, A.; Jankovic, A.; Korac, B. Reactive oxygen, nitrogen, and sulfur species in human male fertility. A crossroad of cellular signaling and pathology. *BioFactors* **2019**. [CrossRef]
41. O'Flaherty, C.; Matsushita-Fournier, D. Reactive oxygen species and protein modifications in spermatozoa. *Biol. Reprod.* **2017**, *97*, 577–585. [CrossRef]
42. López Úbeda, R.; Matas, P.C. An approach to the factors related to sperm capacitation process. *Andrology-Open Access* **2015**, *4*, 128.
43. Jovičić, M.; Pintus, E.; Fenclová, T.; Simoník, O.S.; Chmelíková, E.; Ros-Santaella, J.L.; Sedmíková, M. Effect of nitric oxide on boar sperm motility, membrane integrity, and acrosomal status during semen storage. *Pol. J. Vet. Sci.* **2018**, *21*, 73–82.
44. De Andrade, A.F.C.; Arruda, R.P.; Torres, M.A.; Pieri, N.C.G.; Leite, T.G.; Celeghini, E.C.C.; Oliveira, L.Z.; Gardés, T.P.; Bussiere, M.C.C.; Silva, D.F. Nitric oxide in frozen-thawed equine sperm: Effects on motility, membrane integrity and sperm capacitation. *Anim. Reprod. Sci.* **2018**, *195*, 176–184. [CrossRef] [PubMed]
45. Derbyshire, E.R.; Marletta, M.A. Structure and Regulation of Soluble Guanylate Cyclase. *Annu. Rev. Biochem.* **2012**, *81*, 533–559. [CrossRef] [PubMed]
46. Pan, J.; Yuan, H.; Zhang, X.; Zhang, H.; Xu, Q.; Zhou, Y.; Tan, L.; Nagawa, S.; Huang, Z.X.; Tan, X. Probing the molecular mechanism of human soluble guanylate cyclase activation by no in vitro and in vivo. *Sci. Rep.* **2017**, *7*, 43112. [CrossRef] [PubMed]
47. Sürmeli, N.B.; Müskens, F.M.; Marletta, M.A. The influence of nitric oxide on soluble guanylate cyclase regulation by nucleotides: Role of the pseudosymmetric site. *J. Biol. Chem.* **2015**, *290*, 15570–15580. [CrossRef] [PubMed]
48. Montfort, W.R.; Wales, J.A.; Weichsel, A. Structure and activation of soluble guanylyl cyclase, the nitric oxide sensor. *Antioxid. Redox Signal.* **2017**, *26*, 107–121. [CrossRef] [PubMed]

49. Zamir, N.; Barkan, D.; Keynan, N.; Naor, Z.; Breitbart, H. Atrial natriuretic peptide induces acrosomal exocytosis in bovine spermatozoa. *Am. J. Physiol.* **1995**, *269*, E216–E221. [CrossRef]
50. Rotem, R.; Zamir, N.; Keynan, N.; Barkan, D.; Breitbart, H.; Naor, Z. Atrial natriuretic peptide induces acrosomal exocytosis of human spermatozoa. *Am. J. Physiol.* **1998**, *274*, E218–E223. [CrossRef]
51. Wiesner, B.; Weiner, J.; Middendorff, R.; Hagen, V.; Kaupp, U.B.; Weyand, I. Cyclic nucleotide-gated channels on the flagellum control Ca^{2+} entry into sperm. *J. Cell Biol.* **1998**, *142*, 473–484. [CrossRef]
52. Cisneros-Mejorado, A.; Hernández-Soberanis, L.; Islas-Carbajal, M.C.; Sánchez, D. Capacitation and Ca^{2+} influx in spermatozoa: Role of CNG channels and protein kinase G. *Andrology* **2014**, *2*, 145–154. [CrossRef]
53. Singh, A.P.; Rajender, S. CatSper channel, sperm function and male fertility. *Reprod. Biomed. Online* **2015**, *30*, 28–38. [CrossRef]
54. 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*, 522–536. [CrossRef]
55. McVey, M.; Hill, J.; Howlett, A.; Klein, C. Adenylyl cyclase, a coincidence detector for nitric oxide. *J. Biol. Chem.* **1999**, *274*, 18887–18892. [CrossRef]
56. Kaupp, U.B.; Strünker, T. Signaling in sperm: More different than similar. *Trends Cell Biol.* **2017**, *27*, 101–109. [CrossRef] [PubMed]
57. Gangwar, D.K.; Atreja, S.K. Signalling events and associated pathways related to the mammalian sperm capacitation. *Reprod. Domest. Anim.* **2015**, *50*, 705–711. [CrossRef] [PubMed]
58. Lefièvre, L.; Chen, Y.; Conner, S.J.; Scott, J.L.; Publicover, S.J.; Ford, W.C.L.; Barratt, C.L.R. Human spermatozoa contain multiple targets for protein S-nitrosylation: An alternative mechanism of the modulation of sperm function by nitric oxide? *Proteomics* **2007**, *7*, 3066–3084. [CrossRef] [PubMed]
59. Nagpure, B.V.; Bian, J.S. Interaction of hydrogen sulfide with nitric oxide in the cardiovascular System. *Oxid. Med. Cell. Longev.* **2016**, *2016*, 6904327. [CrossRef]
60. Zhao, Y.; Zhang, W.D.; Liu, X.Q.; Zhang, P.F.; Hao, Y.N.; Li, L.; Chen, L.; Shen, W.; Tang, X.F.; Min, L.J.; et al. Hydrogen sulfide and/or ammonia reduces spermatozoa motility through AMPK/AKT related pathways. *Sci. Rep.* **2016**, *6*, 37884. [CrossRef]
61. Martin-Hidalgo, D.; Hurtado de Llera, A.; Calle-Guisado, V.; Gonzalez-Fernandez, L.; Garcia-Marin, L.; Bragado, M.J. AMPK function in mammalian spermatozoa. *Int. J. Mol. Sci.* **2018**, *19*, E3293. [CrossRef]
62. Xia, Y.Q.; Ning, J.Z.; Cheng, F.; Yu, W.M.; Rao, T.; Ruan, Y.; Yuan, R.; Du, Y. GYY4137 a H_2S donor, attenuates ipsilateral epididymis injury in experimentally varicocele-induced rats via activation of the PI3K/ Akt pathway. *Iran. J. Basic Med. Sci.* **2019**, *22*, 729–735.
63. Yang, G. Gasotransmitters and Protein Post-Translational Modifications. *MOJ Proteomics Bioinform.* **2017**, *5*, 122–124. [CrossRef]
64. Mustafa, A.K.; Gadalla, M.M.; Sen, N.; Kim, S.; Mu, W.; Gazi, S.K.; Barrow, R.K.; Yang, G.; Wang, R.; Snyder, S.H. HS signals through protein S-Sulfhydration. *Sci. Signal.* **2009**, *2*, ra72. [CrossRef] [PubMed]
65. Matsuura, K.; Huang, H.W.; Chen, M.C.; Chen, Y.; Cheng, C.M. Relationship between porcine sperm motility and sperm enzymatic activity using paper-based devices. *Sci. Rep.* **2017**, *7*, 46213. [CrossRef] [PubMed]
66. Wang, L.; Li, Y.; Fu, J.; Zhen, L.; Zhao, N.; Yang, Q.; Li, S.; Li, X. Cadmium inhibits mouse sperm motility through inducing tyrosine phosphorylation in a specific subset of proteins. *Reprod. Toxicol.* **2016**, *63*, 96–106. [CrossRef] [PubMed]
67. Muronetz, V.I.; Kuravsky, M.L.; Barinova, K.V.; Schmalhausen, E.V. Sperm-specific glyceraldehyde-3-phosphate dehydrogenase–an evolutionary acquisition of mammals. *Biochemistry* **2015**, *80*, 1672–1689. [CrossRef] [PubMed]
68. Miki, K.; Qu, W.; Goulding, E.H.; Willis, W.D.; Bunch, D.O.; Strader, L.F.; Perreault, S.D.; Eddy, E.M.; O'Brien, D.A. Glyceraldehyde 3-phosphate dehydrogenase-S, a sperm-specific glycolytic enzyme, is required for sperm motility and male fertility. *Proc. Natl. Acad. Sci. USA* **2004**, *101*, 16501–16506. [CrossRef] [PubMed]
69. Filipovic, M.R.; Zivanovic, J.; Alvarez, B.; Banerjee, R. Chemical Biology of H_2S Signaling through Persulfidation. *Chem. Rev.* **2018**, *118*, 1253–1337. [CrossRef] [PubMed]
70. Lau, N.; Pluth, M.D. Reactive sulfur species (RSS): Persulfides, polysulfides, potential, and problems. *Curr. Opin. Chem. Biol.* **2019**, *49*, 1–8. [CrossRef]
71. Paul, B.D.; Snyder, S.H. H_2S: A Novel Gasotransmitter that Signals by Sulfhydration. *Trends Biochem. Sci.* **2015**, *40*, 687–700. [CrossRef]

72. Mishanina, T.V.; Libiad, M.; Banerjee, R. Biogenesis of reactive sulfur species for signaling by hydrogen sulfide oxidation pathways. *Nat. Chem. Biol.* **2015**, *11*, 457–464. [CrossRef]
73. Aitken, R.J.; Gibb, Z.; Baker, M.A.; Drevet, J.; Gharagozloo, P. Causes and consequences of oxidative stress in Spermatozoa. *Reprod. Fertil. Dev.* **2016**, *28*, 1–10. [CrossRef]
74. Aitken, R.J. Reactive oxygen species as mediators of sperm capacitation and pathological damage. *Mol. Reprod. Dev.* **2017**, *84*, 1039–1052. [CrossRef] [PubMed]
75. Kothari, S.; Thompson, A.; Agarwal, A.; du Plessis, S.S. Free radicals: Their beneficial and detrimental effects on sperm function. *Indian J. Exp. Biol.* **2010**, *48*, 425–435. [PubMed]
76. Jacob, C.; Lancaster, J.R.; Giles, G.I. Reactive sulphur species in oxidative signal transduction. *Biochem. Soc. Trans.* **2004**, *32*, 1015–1017. [CrossRef] [PubMed]
77. Sancho, S.; Vilagran, I. The boar ejaculate: Sperm function and seminal plasma analyses. In *Boar Reproduction*; Bonet, S., Casas, I., Holt, W.V., Bonet, S., Casas, I., Holt, W.V., Yeste, M., Eds.; Springer: Berlin/Heidelberg, Germany, 2013; Chapter 9; pp. 471–516.
78. O'Flaherty, C. The enzymatic antioxidant system of human spermatozoa. *Adv. Androl.* **2014**, *2014*, 626374. [CrossRef]
79. Moretti, E.; Collodel, G.; Fiaschi, A.I.; Micheli, L.; Iacoponi, F.; Cerretani, D. Nitric oxide, malondialdheyde and non-enzymatic antioxidants assessed in viable spermatozoa from selected infertile men. *Reprod. Biol.* **2017**, *17*, 370–375. [CrossRef] [PubMed]
80. Guthrie, H.D.; Welch, G.R.; Long, J.A. Mitochondrial function and reactive oxygen species action in relation to boar motility. *Theriogenology* **2008**, *70*, 1209–1215. [CrossRef]
81. Weidinger, A.; Kozlov, A.V. Biological activities of reactive oxygen and nitrogen species: Oxidative stress versus signal transduction. *Biomolecules* **2015**, *5*, 472–484. [CrossRef]
82. Alizadeh, N.; Abbasi, M.; Abolhassani, F.; Amidi, F.; Mahmoudi, R.; Hoshino, Y.; Sato, E. Effects of aminoguanidine on infertile varicocelized rats: A functional and morphological study. *Daru* **2010**, *18*, 51–56.
83. Pintus, E.; Kadlec, M.; Jovičić, M.; Sedmíková, M.; Ros-Santaella, J.L. Aminoguanidine protects boar spermatozoa against the deleterious effects of oxidative stress. *Pharmaceutics* **2018**, *10*, E212. [CrossRef]
84. Radi, R. Oxygen radicals, nitric oxide, and peroxynitrite: Redox pathways in molecular medicine. *Proc. Natl. Acad. Sci. USA* **2018**, *115*, 5839–5848. [CrossRef]
85. Speckmann, B.; Steinbrenner, H.; Grune, T.; Klotz, L.O. Peroxynitrite: From interception to signaling. *Arch. Biochem. Biophys.* **2016**, *595*, 153–160. [CrossRef] [PubMed]
86. Ighodaro, O.M.; Akinloye, O.A. First line defence antioxidants-superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX): Their fundamental role in the entire antioxidant defence grid. *Alexandria J. Med.* **2018**, *54*, 287–293. [CrossRef]
87. Uribe, P.; Boguen, R.; Treulen, F.; Sänchez, R.; Villegas, J.V. Peroxynitrite-mediated nitrosative stress decreases motility and mitochondrial membrane potential in human spermatozoa. *Mol. Hum. Reprod.* **2014**, *21*, 237–243. [CrossRef] [PubMed]
88. Uribe, P.; Treulen, F.; Boguen, R.; Sánchez, R.; Villegas, J.V. Nitrosative stress by peroxynitrite impairs ATP production in human spermatozoa. *Andrologia* **2017**, *49*, e12615. [CrossRef] [PubMed]
89. Cabrillana, M.E.; Uribe, P.; Villegas, J.V.; Álvarez, J.; Sánchez, R.; Fornés, M.W. Thiol oxidation by nitrosative stress: Cellular localization in human spermatozoa. *Syst. Biol. Reprod. Med.* **2016**, *62*, 325–334. [CrossRef]
90. Uribe, P.; Villegas, J.V.; Cabrillana, M.E.; Boguen, R.; Sánchez, R.; Fornés, M.W.; Isachenko, V.; Isachenko, E. Impact of peroxynitrite-mediated nitrosative stress on human sperm cells. *Free Radic. Biol. Med.* **2018**, *120*, S54. [CrossRef]
91. Serrano, R.; Garrido, N.; Céspedes, J.A.; González-Fernández, L.; García-Marín, L.J.; Bragado, M.J. Molecular mechanisms involved in the impairment of boar sperm motility by peroxynitrite-induced nitrosative stress. *Int. J. Mol. Sci.* **2020**, *21*, E1208. [CrossRef]
92. Giles, G.I.; Nasim, M.J.; Ali, W.; Jacob, C. The reactive sulfur species concept: 15 years on. *Antioxidants* **2017**, *6*, 38. [CrossRef]
93. Szabo, C.; Ransy, C.; Módis, K.; Andriamihaja, M.; Murghes, B.; Coletta, C.; Olah, G.; Yanagi, K.; Bouillaud, F. Regulation of mitochondrial bioenergetic function by hydrogen sulfide. Part I. Biochemical and physiological mechanisms. *Br. J. Pharmacol.* **2014**, *171*, 2099–2122. [CrossRef]
94. Li, L.; Rose, P.; Moore, P.K. Hydrogen sulfide and cell signaling. *Annu. Rev. Pharmacol. Toxicol.* **2011**, *51*, 169–187. [CrossRef]

95. Xie, Z.Z.; Liu, Y.; Bian, J.S. Hydrogen sulfide and cellular redox homeostasis. *Oxid. Med. Cell. Longev.* **2016**, *2016*, 6043038. [CrossRef] [PubMed]
96. Li, G.; Xie, Z.Z.; Chua, J.M.W.; Wong, P.C.; Bian, J. Hydrogen sulfide protects testicular germ cells against heat-induced injury. *Nitric Oxide Biol. Chem.* **2015**, *46*, 165–171. [CrossRef] [PubMed]
97. Ning, J.Z.; Li, W.; Cheng, F.; Rao, T.; Yu, W.M.; Ruan, Y.; Yuan, R.; Zhang, X.B.; Du, Y.; Xiao, C.C. The protective effects of GYY4137 on ipsilateral testicular injury in experimentally varicocele-induced rats. *Exp. Ther. Med.* **2018**, *15*, 433–439. [CrossRef] [PubMed]
98. Jia, Y.; Castellanos, J.; Wang, C.; Sinha-Hikim, I.; Lue, Y.; Swerdloff, R.S.; Sinha-Hikim, A.P. Mitogen-activated protein kinase signaling in male germ cell apoptosis in the rat. *Biol. Reprod.* **2009**, *80*, 771–780. [CrossRef]
99. Porter, A.G.; Jänicke, R.U. Emerging roles of caspase-3 in apoptosis. *Cell Death Differ.* **1999**, *6*, 99–104. [CrossRef] [PubMed]
100. Durairajanayagam, D.; Agarwal, A.; Ong, C. Causes, effects and molecular mechanisms of testicular heat stress. *Reprod. Biomed. Online* **2015**, *30*, 14–27. [CrossRef] [PubMed]
101. Zhang, X.G.; Hong, J.Y.; Yan, G.J.; Wang, Y.F.; Li, Q.W.; Hu, J.H. Association of heat shock protein 70 with motility of frozen-thawed sperm in bulls. *Czech J. Anim. Sci.* **2015**, *60*, 256–262. [CrossRef]
102. Erata, G.Ö.; Koçak Toker, N.; Durlanik, Ö.; Kadioğlu, A.; Aktan, G.; Aykaç Toker, G. The role of heat shock protein 70 (Hsp 70) in male infertility: Is it a line of defense against sperm DNA fragmentation? *Fertil. Steril.* **2008**, *90*, 322–327. [CrossRef]
103. Reddy, V.S.; Yadav, B.; Yadav, C.L.; Anand, M.; Swain, D.K.; Kumar, D.; Kritania, D.; Madan, A.K.; Kumar, J.; Yadav, S. Effect of sericin supplementation on heat shock protein 70 (HSP70) expression, redox status and post thaw semen quality in goat. *Cryobiology* **2018**, *84*, 33–39. [CrossRef]
104. Hancock, J.T.; Whiteman, M. Hydrogen sulfide signaling: Interactions with nitric oxide and reactive oxygen species. *Ann. N. Y. Acad. Sci.* **2016**, *1365*, 5–14. [CrossRef]
105. Almog, T.; Naor, Z. Mitogen activated protein kinases (MAPKs) as regulators of spermatogenesis and spermatozoa functions. *Mol. Cell Endocrinol.* **2008**, *282*, 39–44. [CrossRef] [PubMed]
106. Lee, N.P.Y.; Cheng, C.Y. Nitric oxide/nitric oxide synthase, spermatogenesis, and tight junction dynamics. *Biol. Reprod.* **2004**, *70*, 267–276. [CrossRef] [PubMed]
107. Silva, J.V.; Freitas, M.J.; Correia, B.R.; Korrodi-Gregório, L.; Patrício, A.; Pelech, S.; Fardilha, M. Profiling signaling proteins in human spermatozoa: Biomarker identification for sperm quality evaluation. *Fertil. Steril.* **2015**, *104*, 845–856. [CrossRef] [PubMed]
108. Schieke, S.M.; Briviba, K.; Klotz, L.O.; Sies, H. Activation pattern of mitogen-activated protein kinases elicited by peroxynitrite: Attenuation by selenite supplementation. *FEBS Lett.* **1999**, *448*, 301–303. [CrossRef]
109. Molina, L.C.P.; Luque, G.M.; Balestrini, P.A.; Marín-Briggiler, C.I.; Romarowski, A.; Buffone, M.G. Molecular basis of human sperm capacitation. *Front. Cell Dev. Biol.* **2018**, *6*, 1–23. [CrossRef]
110. Miki, K.; Clapham, D.E. Rheotaxis guides mammalian sperm. *Curr. Biol.* **2013**, *23*, 443–452. [CrossRef]
111. Miraglia, E.; Rullo, M.L.; 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*, 1059–1063. [CrossRef]
112. Wiliński, B.; Wiliński, J.; Gajda, M.; Jasek, E.; Somogyi, E.; Głowacki, M.; Śliwa, L. Sodium hydrosulfide exerts a transitional attenuating effect on spermatozoa migration in vitro. *Folia Biol.* **2015**, *63*, 145–149. [CrossRef]
113. Xia, J.; Ren, D. The BSA-induced Ca^{2+} influx during sperm capacitation is CATSPER channel-dependent. *Reprod. Biol. Endocrinol.* **2009**, *7*, 119. [CrossRef]
114. Schiffer, C.; Rieger, S.; Brenker, C.; Young, S.; Hamzeh, H.; Wachten, D.; Tüttelmann, F.; Röpke, A.; Kaupp, U.B.; Wang, T.; et al. Rotational motion and rheotaxis of human sperm do not require functional CatSper channels and transmembrane Ca^{2+} signaling. *EMBO J.* **2020**, *39*, e102363. [CrossRef]
115. Gupta, R.K.; Swain, D.K.; Singh, V.; Anand, M.; Choudhury, S.; Yadav, S.; Saxena, A.; Garg, S.K. Molecular characterization of voltage-gated potassium channel (Kv) and its importance in functional dynamics in bull spermatozoa. *Theriogenology* **2018**, *114*, 229–236. [CrossRef] [PubMed]
116. Björkgren, I.; Lishko, P.V. Fertility and trp channels. In *Neurobiology of TRP Channels*; Emir, T.L.R., Ed.; CRC Press/Taylor & Francis: Boca Raton, FL, USA, 2017.
117. Kumar, A.; Mishra, A.K.; Swain, D.K.; Singh, V.; Yadav, S.; Saxena, A. Role of transient receptor potential channels in regulating spermatozoa functions: A mini-review. *Vet. World* **2018**, *11*, 1618–1623. [CrossRef] [PubMed]

118. Yoshida, T.; Inoue, R.; Morii, T.; Takahashi, N.; Yamamoto, S.; Hara, Y.; Tominaga, M.; Shimizu, S.; Sato, Y.; Mori, Y. Nitric oxide activates TRP channels by cysteine S-nitrosylation. *Nat. Chem. Biol.* **2006**, *2*, 596–607. [CrossRef] [PubMed]
119. Mundt, N.; Spehr, M.; Lishko, P.V. TRPV4 is the temperature-sensitive ion channel of human sperm. *Elife* **2018**, *7*, e35853. [CrossRef] [PubMed]
120. Kumar, A.; Mishra, A.K.; Singh, V.; Yadav, S.; Saxena, A.; Garg, S.K.; Swain, D.K. Molecular and functional insights into transient receptor potential vanilloid 1 (TRPV1) in bull spermatozoa. *Theriogenology* **2019**, *128*, 207–217. [CrossRef] [PubMed]
121. Bernabò, N.; Pistilli, M.G.; Mattioli, M.; Barboni, B. Role of TRPV1 channels in boar spermatozoa acquisition of fertilizing ability. *Mol. Cell. Endocrinol.* **2010**, *323*, 224–231. [CrossRef] [PubMed]
122. Claudia, C.; Horatiu, S.; Iudith, I.; Vasile, B.; Constanta, S. Research regarding the role of TRPV1 and capsaicin (CPS) implication for capacitation and acrosome reaction. *Rom. Biotechnol. Lett.* **2014**, *19*, 9437–9441.
123. King, A.L.; Polhemus, D.J.; Bhushan, S.; Otsuka, H.; Kondo, K.; Nicholson, C.K.; Bradley, J.M.; Islam, K.N.; Calvert, J.W.; Tao, Y.X. Hydrogen sulfide cytoprotective signaling is endothelial nitric oxide synthase-nitric oxide dependent. *Proc. Natl. Acad. Sci. USA* **2014**, *111*, 3182–3187. [CrossRef]
124. Di, T.; Sullivan, J.A.; Magness, R.R.; Zhang, L.; Bird, I.M. Pregnancy-specific enhancement of agonist-stimulated ERK-1/2 signaling in uterine artery endothelial cells increases Ca^{2+} sensitivity of endothelial nitric oxide synthase as well as cytosolic phospholipase A_2. *Endocrinology* **2001**, *142*, 3014–3026. [CrossRef]
125. Cai, H.; Li, Z.; Davis, M.E.; Kanner, W.; Harrison, D.G.; Dudley, S.C. Akt-dependent phosphorylation of serine 1179 and mitogen-activated protein kinase kinase/extracellular signal-regulated kinase 1/2 cooperatively mediate activation of the endothelial nitric-oxide synthase by hydrogen peroxide. *Mol. Pharmacol.* **2003**, *63*, 325–331. [CrossRef]
126. Benetti, L.R.; Campos, D.; Gurgueira, S.A.; Vercesi, A.E.; Guedes, C.E.V.; Santos, K.L.; Wallace, J.L.; Teixeira, S.A.; Florenzano, J.; Costa, S.K.P.; et al. Hydrogen sulfide inhibits oxidative stress in lungs from allergic mice in vivo. *Eur. J. Pharmacol.* **2013**, *698*, 463–469. [CrossRef] [PubMed]
127. Kolluru, G.K.; Yuan, S.; Shen, X.; Kevil, C.G. H_2S regulation of nitric oxide metabolism. *Methods Enzymol.* **2015**, *554*, 271–297. [PubMed]
128. Ivanovic-Burmazovic, I.; Filipovic, M.R. Saying NO to H_2S: A Story of HNO, HSNO, and $SSNO^-$. *Inorg. Chem.* **2019**, *58*, 4039–4051. [CrossRef] [PubMed]
129. Cortese-Krott, M.M.; Kuhnle, G.G.C.; Dyson, A.; Fernandez, B.O.; Grman, M.; DuMond, J.F.; Barrow, M.P.; McLeod, G.; Nakagawa, H.; Ondrias, K.; et al. Key bioactive reaction products of the NO/H_2S interaction are S/N-hybrid species, polysulfides, and nitroxyl. *Proc. Natl. Acad. Sci. USA* **2015**, *112*, E4651–E4660. [CrossRef]
130. Filipovic, M.R.; Miljkovic, J.L.; Nauser, T.; Royzen, M.; Klos, K.; Shubina, T.; Koppenol, W.H.; Lippard, S.J.; Ivanović-Burmazović, I. Chemical characterization of the smallest S-nitrosothiol, HSNO; Cellular cross-talk of H_2S and S-nitrosothiols. *J. Am. Chem. Soc.* **2012**, *134*, 12016–12027. [CrossRef]
131. Andrews, K.L.; Lumsden, N.G.; Farry, J.; Jefferis, A.M.; Kemp-Harper, B.K.; Chin-Dusting, J.P.F. Nitroxyl: A vasodilator of human vessels that is not susceptible to tolerance. *Clin. Sci.* **2015**, *129*, 179–187. [CrossRef]
132. Bianco, C.L.; Toscano, J.P.; Bartberger, M.D.; Fukuto, J.M. The chemical biology of HNO signaling. *Arch. Biochem. Biophys.* **2017**, *617*, 129–136. [CrossRef]
133. Jackson, M.I.; Fields, H.F.; Lujan, T.S.; Cantrell, M.M.; Lin, J.; Fukuto, J.M. The effects of nitroxyl (HNO) on H_2O_2 metabolism and possible mechanisms of HNO signaling. *Arch. Biochem. Biophys.* **2013**, *538*, 120–129. [CrossRef]
134. Broniowska, K.A.; Diers, A.R.; Hogg, N. S-Nitrosoglutathione. *Biochim. Biophys. Acta* **2013**, *1830*, 3173–3181. [CrossRef]
135. Ondrias, K.; Stasko, A.; Cacanyiova, S.; Sulova, Z.; Krizanova, O.; Kristek, F.; Malekova, L.; Knezl, V.; Breier, A. H_2S and HS^- donor NaHS releases nitric oxide from nitrosothiols, metal nitrosyl complex, brain homogenate and murine L1210 leukaemia cells. *Pflug. Arch. Eur. J. Physiol.* **2008**, *457*, 271–279. [CrossRef]
136. Kumar, M.R.; Farmer, P.J. Characterization of polysulfides, polysulfanes, and other unique species in the reaction between GSNO and H_2S. *Molecules* **2019**, *24*, E3090. [CrossRef] [PubMed]
137. Berenyiova, A.; Grman, M.; Mijuskovic, A.; Stasko, A.; Misak, A.; Nagy, P.; Ondriasova, E.; Cacanyiova, S.; Brezova, V.; Feelisch, M.; et al. The reaction products of sulfide and S-nitrosoglutathione are potent vasorelaxants. *Nitric Oxide Biol. Chem.* **2015**, *46*, 123–130. [CrossRef] [PubMed]

138. Musset, B.; Clark, R.A.; DeCoursey, T.E.; Petheo, G.L.; Geiszt, M.; Chen, Y.; Cornell, J.E.; Eddy, C.A.; Brzyski, R.G.; El Jamali, A. NOX5 in human spermatozoa: Expression, function, and regulation. *J. Biol. Chem.* **2012**, *287*, 9376–9388. [CrossRef] [PubMed]
139. Cortese-Krott, M.M.; Fernandez, B.O.; Santos, J.L.T.; Mergia, E.; Grman, M.; Nagy, P.; Kelm, M.; Butler, A.; Feelisch, M. Nitrosopersulfide (SSNO-) accounts for sustained NO bioactivity of S-nitrosothiols following reaction with sulfide. *Redox Biol.* **2014**, *2*, 234–244. [CrossRef]
140. Cortese-Krott, M.M.; Fernandez, B.O.; Kelm, M.; Butler, A.R.; Feelisch, M. On the chemical biology of the nitrite/sulfide interaction. *Nitric Oxide Biol. Chem.* **2015**, *46*, 14–24. [CrossRef]
141. Wedmann, R.; Ivanovic-Burmazovic, I.; Filipovic, M.R. Nitrosopersulfide ($SSNO^-$) decomposes in the presence of sulfide, cyanide or glutathione to give $HSNO/SNO^-$: Consequences for the assumed role in cell signalling. *Interface Focus* **2017**, *7*, 20160139. [CrossRef]

SCIENTIFIC REPORTS
nature research

OPEN

Divergent effect of fast- and slow-releasing H_2S donors on boar spermatozoa under oxidative stress

Eliana Pintus*, Marija Jovičić, Martin Kadlec & José Luis Ros-Santaella*

Hydrogen sulphide (H_2S) is involved in the physiology and pathophysiology of different cell types, but little is known about its role in sperm cells. Because of its reducing properties, we hypothesise that H_2S protects spermatozoa against the deleterious effects of oxidative stress, a condition that is common to several male fertility disorders. This study aimed i) to determine the total antioxidant capacities of Na_2S and GYY4137, which are fast- and slow-releasing H_2S donors, respectively, and ii) to test whether H_2S donors are able to protect spermatozoa against oxidative stress. We found that Na_2S and GYY4137 show different antioxidant properties, with the total antioxidant capacity of Na_2S being mostly unstable and even undetectable at 150 µM. Moreover, both H_2S donors preserve sperm motility and reduce acrosome loss, although the effects were both dose and donor dependent. Within the range of concentrations tested (3–300 µM), GYY4137 showed positive effects on sperm motility, whereas Na_2S was beneficial at the lowest concentration but detrimental at the highest. Our findings show that Na_2S and GYY4137 have different antioxidant properties and suggest that both H_2S donors might be used as *in vitro* therapeutic agents against oxidative stress in sperm cells, although the optimal therapeutic range differs between the compounds.

Hydrogen sulphide (H_2S) is the most recently discovered gaseous molecule that participates in a variety of biological functions, as do nitric oxide (NO) and carbon monoxide (CO). In mammals, H_2S can be synthesised by enzymatic or non-enzymatic pathways[1]. Overall, it seems likely that most of the H_2S produced within an organism is generated by the H_2S-synthesising enzymes: cystathionine γ-lyase (CSE), cystathionine β-synthase (CBS), and 3-mercaptopyruvate sulphurtransferase (3-MST), with the latter coupled with cysteine aminotransferase (CAT)[2].

In the male reproductive system, the expression of H_2S-generating enzymes has been reported in the testis[3,4], epididymis[5], penile corpus cavernosum[6], and spermatozoa[7,8], which strongly suggests that this gasotransmitter is involved in sperm physiology to some extent. In a recent study, Wang *et al.* found that asthenospermic men show reduced levels of H_2S in their seminal plasma and that exogenous H_2S supplementation improves their sperm motility[8]. In contrast, in boar spermatozoa, H_2S exerts no or negative effects on sperm motility, viability, and mitochondrial membrane potential[9]. With both positive and negative effects documented, there is still controversy concerning the role of H_2S in sperm cells. This apparent discrepancy might, at least partly, be a result of H_2S dose- and donor-dependent effects[10].

According to their chemical structure and source, H_2S donors include inorganic salts and derivatives of phosphorodithioate, garlic extracts, thioaminoacids, and anti-inflammatory drugs[11]. On the basis of their release mechanism, H_2S donors can be classified in two categories: slow- and fast-releasing agents. Among the fast-releasing H_2S donors, the inorganic salts sodium sulphide (Na_2S) and sodium hydrosulphide (NaHS) are probably most frequently employed in biological studies. Both salts can be dissolved in aqueous solution, leading to an instantaneous release of H_2S that mimics a bolus administration. Despite the common use of these donors in experimental studies, it is becoming increasingly clear that their gas release might not be representative of the physiological H_2S levels in tissues and cells[12]. On the other hand, slow-releasing H_2S donors, like the phosphorodithioate derivative GYY4137, produce a slow and continuous release of gas, which is more similar to the physiological conditions found within organisms[12]. For this reason, the use of different H_2S donors in studies is useful to elucidate the biological activity and possible therapeutic effects[12].

Department of Veterinary Sciences, Faculty of Agrobiology, Food and Natural Resources, Czech University of Life Sciences Prague, Kamýcká 129, 165 00, Praha, 6-Suchdol, Czech Republic. *email: eliana.pintus27@gmail.com; rossantaella@gmail.com

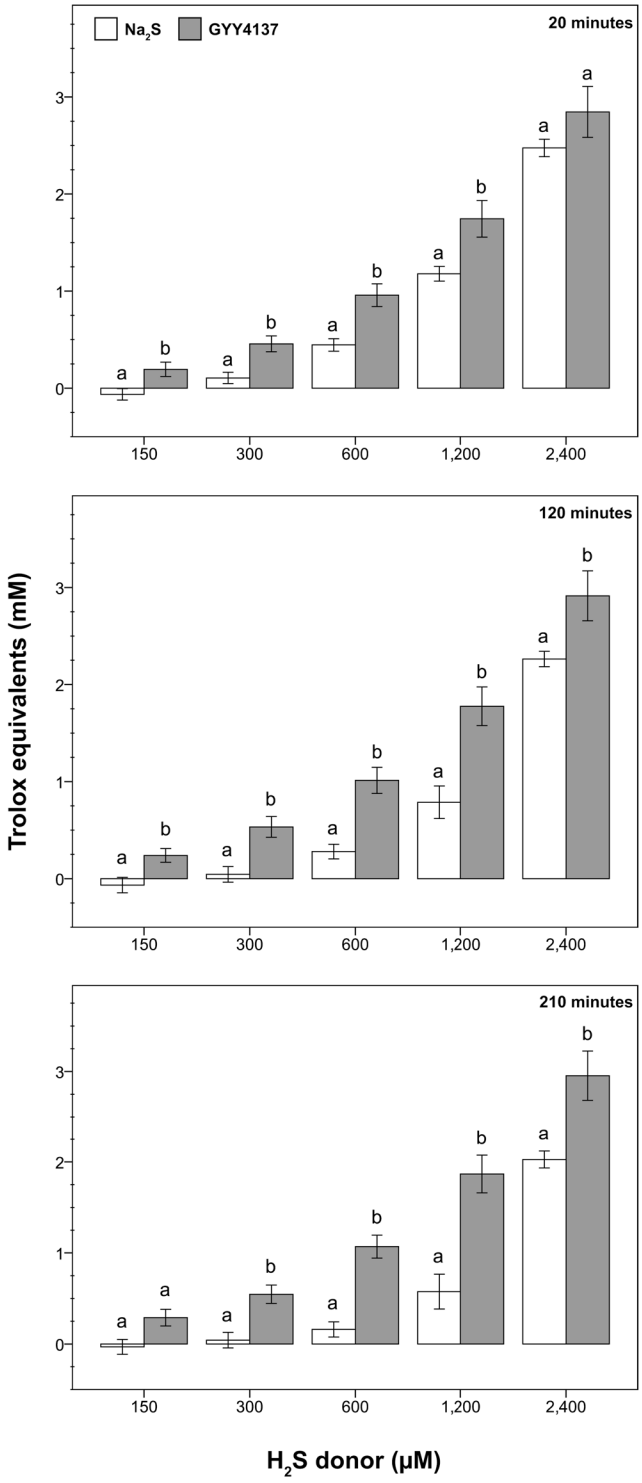


Figure 1. Total antioxidant capacity and stability of the H_2S donors Na_2S and GYY4137. The H_2S donors were incubated in phosphate-buffered saline solution at 38 °C in a water bath. Total antioxidant capacity is expressed as Trolox equivalents (mM). White histograms: Na_2S; grey histograms: GYY4137. Upper panel: 20 minutes of incubation; middle panel: 120 minutes of incubation; lower panel: 210 minutes of incubation. Different letters indicate significant differences ($p < 0.05$) between H_2S donors at the same concentration and incubation time. Data are shown as the mean±standard error of four replicates.

By virtue of its activity as a reducing agent, H_2S attenuates the damage induced by oxidative stress in different cells and tissues (e.g. neurons[13], gastric cells[14], lung cells[15]). Oxidative stress is an underlying condition common to several male reproductive disorders, in which high levels of reactive oxygen species (ROS) cause sperm dysfunction (e.g. decreased sperm motility, impaired membrane and DNA integrity, increased lipid peroxidation)

and infertility[16,17]. Previous studies have shown that H_2S is able to alleviate the effects of oxidative stress on testicular functions[4,8,18], but the *in vitro* effects of this gasotransmitter on sperm cells under a ROS-generating system still need to be elucidated.

The aim of this study was to evaluate the total antioxidant capacity and stability of the H_2S donors Na_2S and GYY4137 under standard conditions (38 °C, pH≈7) and at different times (i.e. 20, 120, and 210 minutes) during the incubation (experiment I). Because sperm motility under a ROS-generating system may drop in a few hours[19,20] and based on the opposite modalities of H_2S release by Na_2S and GYY4137 (i.e. fast and slow release, respectively), these incubation times were chosen to determine the dynamics of the antioxidant activity of each donor during the early, mid, and late stages of incubation. Based on the results from experiment I and the physiological total antioxidant capacity of boar seminal plasma[21], we then established a suitable range of concentrations of Na_2S and GYY4137 to be tested in boar sperm samples under a ROS-generating system (experiment II). Although some Na_2S and GYY4137 concentrations used in experiment II show a total antioxidant capacity that is below the range of detection by spectrophotometry, they were included in our experimental design because increasing evidence suggests that *in vivo* H_2S levels range from low µM to high nM[22]. Next, we evaluated the effects of both donors on sperm motility, mitochondrial activity, plasma membrane integrity, acrosomal status, and lipid peroxidation. The results from this study elucidate the role of H_2S donors in sperm samples under oxidative stress and the possible therapeutic implications of these compounds for alleviating the negative effects of ROS on sperm function.

Results

Experiment I. Total antioxidant capacity and stability of H_2S donors. As can be seen from Table 1, Na_2S and GYY4137 showed different total antioxidant capacities and stabilities during the incubation. Overall, the total antioxidant capacity of Na_2S significantly decreased between 20 and 210 minutes of incubation, whereas that of GYY4137 tended to increase during this period and was significantly higher after 210 minutes than after 20 minutes of incubation at 2,400 and 1,200 µM ($p < 0.05$). Moreover, GYY4137 showed detectable levels of total antioxidant capacity at all concentrations tested, whereas Na_2S was unstable within the range of 300 to 1,200 µM and was undetectable at 150 µM. Irrespective of the concentration considered, GYY4137 showed greater total antioxidant capacity than Na_2S (Fig. 1).

Experiment II. Effect of H_2S donors on boar sperm parameters under induced oxidative stress. *Sperm motility.* Overall, the effects of H_2S on boar sperm motility under induced oxidative stress were dose- and donor-dependent (Table 2, Fig. 2). Thus, 3 and 30 µM GYY4137 and 3 µM Na_2S preserved the sperm motility and kinetics under the ROS-generating system. Interestingly, all of the sperm kinetic parameters in these treatments did not differ from those of the control group without oxidative stress (CTR; $p > 0.05$). The results with both 3 and 30 µM GYY4137 showed higher percentages of total motility (TM) relative to that of the control group under oxidative stress (CTR-ox; $p < 0.01$). Moreover, a dose of 30 µM GYY4137 significantly increased the percentage of progressive motility (PM) over that in the CTR-ox group ($p = 0.040$). Although differences were not statistically significant, higher average path velocity (VAP) and straight-line velocity (VSL) were observed in samples treated with 3 µM GYY4137 than in those in the CTR-ox group ($p = 0.071$ and $p = 0.064$, respectively). On the other hand, the effects of Na_2S were markedly dose dependent. At 300 µM, this fast-releasing H_2S donor showed clear negative effects on sperm motility. No motile spermatozoa were observed in any replicate; therefore, no kinetics data could be provided. By contrast, at the lowest concentration, Na_2S significantly increased the percentage of motile sperm cells relative to that in the CTR-ox group ($p = 0.018$). At a concentration of 30 µM, Na_2S greatly decreased the TM, PM, VAP, and VSL ($p < 0.05$), although it did not affect the curvilinear velocity (VCL) and the remaining motion parameters in comparison with those of the CTR-ox group ($p > 0.05$). However, at this Na_2S concentration, we observed some variability among the replicates with the percentage of motile spermatozoa ranging from 0 to almost 30%. There were no differences between the CTR-ox and H_2S donor treatments in the amplitude of lateral head displacement (ALH), beat-cross frequency (BCF), linearity (LIN), straightness (STR), and wobble (WOB; $p > 0.05$).

Sperm mitochondrial status. At a concentration of 300 µM, Na_2S showed clear negative effects on boar sperm mitochondrial status. In every replicate, there were no spermatozoa with active mitochondria (Table 3, Fig. 3). There were no differences between the CTR-ox group and the remaining treatment groups ($p > 0.05$).

Sperm plasma membrane integrity and lipid peroxidation. There was no significant effect of GYY4137 on sperm plasma membrane integrity at any of the concentrations used ($p > 0.05$, Table 3). On the other hand, 300 µM Na_2S markedly impaired the plasma membrane integrity relative to the results obtained for the CTR-ox group ($p < 0.001$, Table 3, Fig. 3). No effects were observed at the remaining Na_2S concentrations ($p > 0.05$). A similar pattern was observed for the sperm lipid peroxidation: higher values of malondialdehyde (MDA) per 10^8 spermatozoa were observed in samples treated with 300 µM Na_2S than those in the CTR-ox group and the other treatment groups ($p < 0.01$, Fig. 4). No differences in the MDA levels were found between the CTR-ox group and the remaining treatment groups ($p > 0.05$).

Acrosomal status. We found that 300 µM Na_2S impaired the acrosome integrity (normal apical ridge or NAR test) relative to that of the CTR group, both with and without oxidative stress ($p < 0.001$, Table 3, Fig. 3). No differences were observed in the NAR test results between the CTR-ox group and the other treatment groups ($p > 0.05$). On the other hand, all treatments showed lower percentages of acrosome-lost spermatozoa (evaluated with peanut agglutinin–fluorescein isothiocyanate, PNA-FITC) than that of the CTR-ox group ($p \leq 0.001$, Table 3).

Treatment	Concentration (μM)	Time (min)		
		20	120	210
Na_2S	2,400	2,474.6 ± 89.8^a	2,262.3 ± 79.4^{ab}	2,027.1 ± 92.8^b
	1,200	1,178.1 ± 75.7^a	787.4 ± 168.1^{ab}	575.2 ± 191.3^b
	600	445.4 ± 64.6^a	278.6 ± 75.1^{ab}	160.2 ± 83.3^b
	300	105.5 ± 58.0^a	44.5 ± 80.1^a	42.6 ± 85.2^a
	150	n.d.	n.d.	n.d.
GYY4137	2,400	2,845.9 ± 262.7^a	2,913.8 ± 257.3^{ab}	2,954.2 ± 270.7^b
	1,200	1,745.0 ± 188.8^a	1,775.9 ± 199.1^{ab}	1,867.2 ± 207.3^b
	600	958.3 ± 117.2^a	1,012.7 ± 135.0^a	1,069.6 ± 125.3^a
	300	456.0 ± 81.6^a	532.8 ± 107.2^a	546.0 ± 101.1^a
	150	194.1 ± 73.7^a	239.5 ± 70.5^a	289.4 ± 91.3^a
PBS		n.d.	n.d.	n.d.

Table 1. Total antioxidant capacity and stability of the H_2S donors Na_2S and GYY4137. The H_2S donors were incubated in phosphate-buffered saline solution at 38 °C in a water bath. Total antioxidant capacity is expressed as Trolox equivalents (μM). Different superscripts indicate significant differences ($p < 0.05$) among times within each donor concentration. PBS: phosphate-buffered saline solution; n.d.: not detectable. Data are shown as the mean±standard error of four replicates.

Treatment	Conc. (μM)	Time (min)	TM (%)	PM (%)	VAP (μm/s)	VCL (μm/s)	VSL (μm/s)	ALH (μm)	BCF (Hz)	LIN (%)	STR (%)	WOB (%)
CTR		20	75.6 ± 2.9	52.8 ± 5.8	43.1 ± 1.7	83.8 ± 3.7	34.8 ± 1.6	3.1 ± 0.1	13.5 ± 0.4	41.7 ± 2.8	80.7 ± 2.8	50.1 ± 1.9
CTR		210	73.1 ± 3.4^a	70.8 ± 5.2^{ac}	42.0 ± 2.6^a	69.0 ± 5.1^a	37.8 ± 1.8^a	3.1 ± 0.6^a	15.6 ± 0.6^a	55.8 ± 3.0^a	89.9 ± 2.3^a	61.0 ± 2.3^a
CTR-ox		210	46.7 ± 8.7^c	65.8 ± 2.3^a	33.1 ± 5.4^b	52.7 ± 9.6^{ab}	30.8 ± 4.7^a	2.1 ± 0.4^{bc}	16.3 ± 0.5^a	62.9 ± 2.9^a	93.5 ± 1.2^a	66.4 ± 2.5^a
Na_2S-ox	300	210	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
	30	210	7.9 ± 5.5^d	44.8 ± 5.5^b	21.7 ± 2.0^c	34.7 ± 2.7^b	20.3 ± 2.1^b	1.5 ± 0.1^c	15.1 ± 0.5^a	62.3 ± 1.6^a	94.3 ± 0.7^a	65.6 ± 1.3^a
	3	210	65.6 ± 6.2^{ab}	69.3 ± 5.1^{ac}	39.2 ± 2.6^{ab}	64.7 ± 8.0^a	35.3 ± 1.6^a	2.6 ± 0.2^{ab}	15.7 ± 0.5^a	58.1 ± 4.4^a	90.6 ± 2.8^a	63.0 ± 3.3^a
GYY4137-ox	300	210	57.2 ± 6.9^{bc}	70.5 ± 3.4^{ac}	35.5 ± 4.4^{ab}	56.0 ± 8.2^a	33.0 ± 3.8^a	2.3 ± 0.3^{abc}	16.0 ± 0.3^a	61.8 ± 3.0^a	93.0 ± 1.6^a	65.6 ± 2.4^a
	30	210	69.0 ± 6.2^{ab}	77.2 ± 2.5^c	38.1 ± 3.7^{ab}	59.0 ± 6.7^a	35.6 ± 3.3^a	2.5 ± 0.3^{ab}	16.2 ± 0.4^a	62.2 ± 2.8^a	93.0 ± 1.3^a	66.2 ± 2.2^a
	3	210	69.8 ± 7.7^{ab}	75.3 ± 3.7^{ac}	41.3 ± 4.0^{ab}	65.9 ± 7.5^a	38.0 ± 3.5^a	2.7 ± 0.3^{ab}	16.1 ± 0.5^a	59.3 ± 2.8^a	91.8 ± 1.5^a	63.8 ± 2.2^a

Table 2. Boar sperm motility and kinetics in samples submitted to oxidative stress and supplemented with the H_2S donors Na_2S and GYY4137. Different superscripts within the same column indicate significant differences ($p < 0.05$) among treatments within the same incubation time. Conc.: concentration; TM: total motility; PM: progressive motility; VAP: average path velocity; VCL: curvilinear velocity; VSL: straight-line velocity; ALH: amplitude of lateral head displacement; BCF: beat-cross frequency; LIN: linearity (VSL/VCL); STR: straightness (VSL/VAP); WOB: wobble (VAP/VCL); CTR: control; ox: samples submitted to induced oxidative stress; n.a.: not available. Data are shown as the mean±standard error of six replicates.

Discussion

In this study, we provide the first evidence, to the best of our knowledge, that Na_2S and GYY4137 show different total antioxidant capacities and stabilities under standard conditions (38 °C, physiological pH) and after different periods (20, 120, and 210 minutes) of incubation. Our results also reveal that the total antioxidant capacity of Na_2S is less stable than that of GYY4137, although the latter's total antioxidant capacity tends to increase over time. This phenomenon should be taken into account in studies entailing cell incubation at 38 °C and at physiological pH, because the release of H_2S by GYY4137 is both pH and temperature dependent[12]. Moreover, GYY4137 shows higher total antioxidant capacity than that of Na_2S after any incubation time, with the total antioxidant capacity of Na_2S at 150 μM even being undetectable by spectrophotometry after 20 minutes of incubation. The patterns observed in the antioxidant capacities of these H_2S donors may not reflect their H_2S release, given that the inorganic salts Na_2S and NaHS lead to a larger but shorter gas release than GYY4137[23,24].

Our results show that both H_2S donors partly palliate the damages provoked by oxidative stress in sperm cells, supporting the results found previously in other cells (neurons[13,25], gastric cells[14], testicular germ cells[4]) and organs (lungs[15,26], testes[18]). In these studies, oxidative stress was induced by *in vivo* or *in vitro* ROS-generating systems, such as glutamate, hydrogen peroxide (H_2O_2), heat, tobacco smoke, ovalbumin sensitisation, and ischaemia-reperfusion injury. In our study, we used Fe^{2+}/ascorbate, which induces lipid peroxidation and catalyses the production of hydroxyl radicals (•OH), the most powerful free radical known, by the Fenton reaction[27]. Moreover, several previous studies[4,13–15,18,25,26] employed a single H_2S donor, whereas two H_2S-releasing agents were tested in our study. To date, few studies[8,23,28] have investigated the biological effects of both fast- and slow-releasing H_2S donors in cells under oxidative stress conditions. In agreement with these studies, our findings

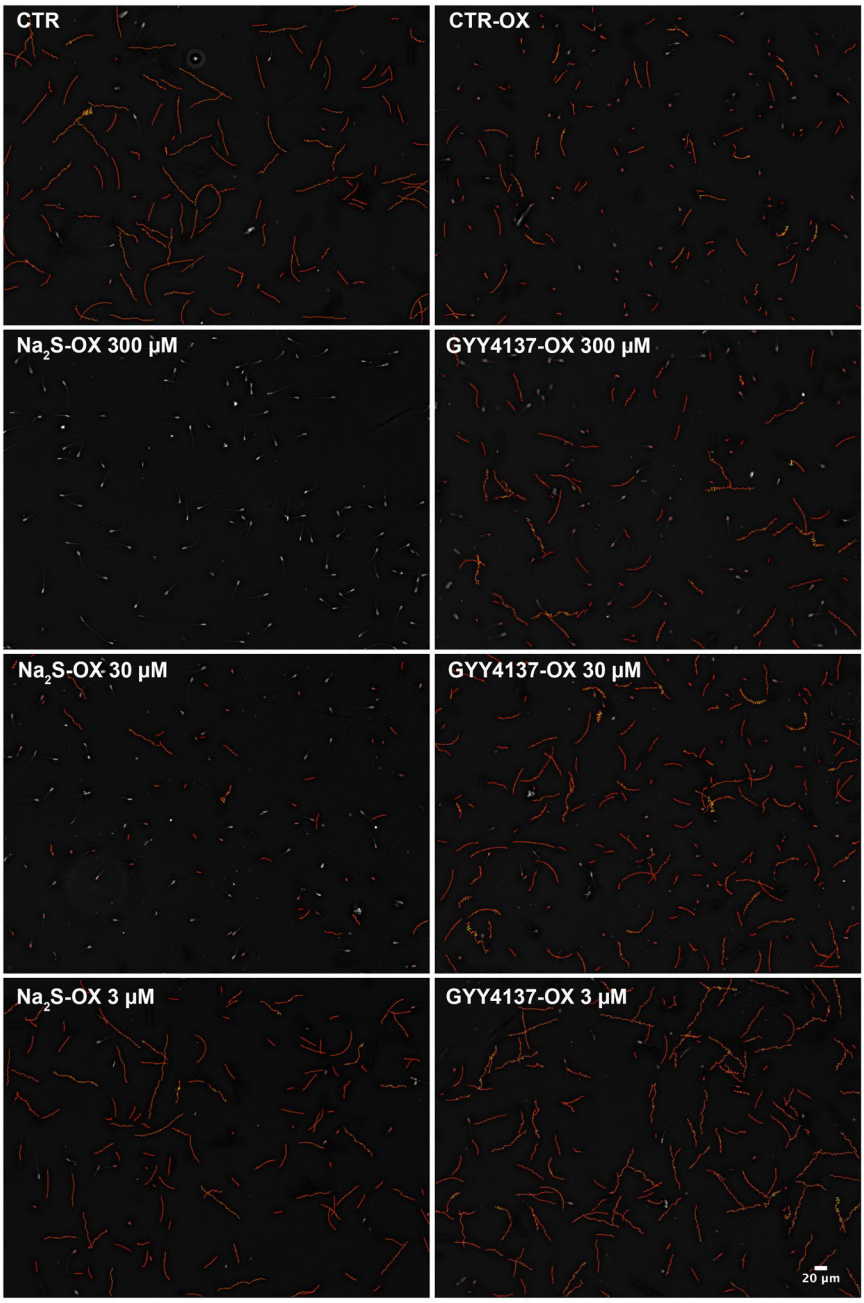


Figure 2. Boar sperm motility in samples submitted to oxidative stress and supplemented with the H_2S donors Na_2S and GYY4137. Representative images of sperm trajectories assessed by Computer-Assisted Sperm Analysis (CASA). Red trajectories show motile spermatozoa (cells are not shown because of overlapping with the trajectories), whereas immotile sperm cells are fully shown.

confirm that the effects of H_2S donors are not only dose but also donor dependent. Moreover, for the first time to the best of our knowledge, the *in vitro* effects of fast- and slow-releasing H_2S donors were evaluated in sperm cells in the presence of a ROS-generating system. Overall, we found that the slow-releasing H_2S donor GYY4137 did not show any cytotoxic effect. Moreover, 3 and 30 µM GYY4137 preserved the boar sperm motility against the detrimental effects of oxidative stress. Interestingly, at both concentrations, the percentage of motile sperm cells was almost 50% higher than that of the CTR-ox samples and no kinetic parameters differed from those of the CTR group without oxidative stress. Moreover, 30 µM GYY4137 samples showed a higher percentage of progressive motility than those in the CTR-ox group. However, in contrast to other studies[8,18], we did not find any effect of GYY4137 on the levels of lipid peroxidation, which may be due to the different cell type and donor concentration used in our study. Our findings also show that, in comparison with GYY4137, the effects of Na_2S were markedly dose dependent. At a concentration of 3 µM, this fast-releasing H_2S donor preserves boar sperm motility (40.5% higher than that in the CTR-ox samples), whereas it shows clear cytotoxic effects at 300 µM. This

Treatment	Conc. (μM)	Time (min)	Active mitochondria (%)	Intact plasma membrane (%)	Intact acrosome (NAR, %)	Acrosome loss (PNA, %)
CTR		20	56.0 ± 3.2	82.0 ± 2.2	95.1 ± 0.9	1.4 ± 0.3
CTR		210	62.8 ± 3.3^{a}	76.6 ± 2.3^{a}	94.8 ± 1.0^{a}	2.4 ± 0.2^{a}
CTR-ox		210	62.4 ± 1.2^{a}	67.8 ± 3.9^{abc}	92.9 ± 0.8^{a}	3.9 ± 0.7^{b}
Na_2S-ox	300	210	0^{b}	21.2 ± 6.0^{d}	31.1 ± 5.6^{b}	2.0 ± 0.3^{a}
	30	210	60.8 ± 5.1^{a}	66.1 ± 4.7^{bc}	94.6 ± 0.7^{a}	2.2 ± 0.4^{a}
	3	210	62.1 ± 3.3^{a}	74.8 ± 3.3^{abc}	92.8 ± 0.6^{a}	1.7 ± 0.4^{a}
GYY4137-ox	300	210	67.7 ± 2.8^{a}	70.8 ± 3.8^{abc}	93.0 ± 1.4^{a}	1.5 ± 0.4^{a}
	30	210	62.9 ± 2.9^{a}	75.8 ± 3.2^{a}	94.1 ± 0.9^{a}	2.3 ± 0.4^{a}
	3	210	63.1 ± 1.8^{a}	76.5 ± 3.6^{a}	94.2 ± 0.6^{a}	1.8 ± 0.4^{a}

Table 3. Boar sperm mitochondrial status, plasma membrane integrity, and acrosomal status in samples submitted to oxidative stress and supplemented with the H_2S donors Na_2S and GYY4137. Different superscripts within the same column indicate significant differences ($p < 0.05$) among treatments within the same incubation time. Conc.: concentration; NAR: normal apical ridge; PNA: peanut agglutinin-fluorescein isothiocyanate; CTR: control; ox: samples submitted to induced oxidative stress. Data are shown as the mean±standard error of six replicates.

pattern may reflect the well-known biphasic biological dose–response curve of H_2S: it acts as an antioxidant at low concentrations, but a pro-oxidant at high concentrations[29]. Interestingly, we also found that 30 μM Na_2S was not cytotoxic (i.e. no effect was observed on the mitochondrial activity, plasma membrane integrity, acrosome integrity, or lipid peroxidation), but it strongly inhibited sperm motility. As in our findings, Zhao *et al.* found that 25 μM Na_2S inhibits boar sperm motility although it does not affect the viability and mitochondrial membrane potential[9]. Likewise, NaHS has been shown to inhibit the motility of mouse and human spermatozoa[8,30]. In our study, the inhibitory effects of Na_2S on sperm motility were described by using a comprehensive set of kinetic parameters provided by Computer-Assisted Sperm Analysis (CASA). Overall, 30 μM Na_2S remarkably decreased the sperm motility, VAP, and VSL, but it did not affect the remaining motion parameters. In spite of some variability among replicates, a small percentage of sperm cells showed very slow but progressive movement. With regard to the mechanism of action, Zhao *et al.* found that the inhibitory effects of Na_2S on sperm motility are related to the adenosine 5′-monophosphate-activated protein kinase (AMPK) and protein kinase B (AKT) pathways[9], whereas a more recent study revealed that H_2S promotes the secretion of K^+ in the epididymis; this suppresses sperm motility and may contribute to keeping the sperm cells in a quiescent state before ejaculation[5]. Another plausible explanation for the reduced sperm motility elicited by Na_2S can be provided by the inhibitory effect of H_2S on cytochrome *c* oxidase (complex IV), the final component of the electron transport chain that plays a key role in aerobic respiration and adenosine triphosphate (ATP) generation[29]. Because mammalian spermatozoa devote most of the energy generated as intracellular ATP to motility[31], a reduction in ATP levels would lead to an immediate decrease in sperm motility. In this way, for instance, cardiac cells treated with 25 μM Na_2S showed over 80% decreased O_2 consumption rate relative to the baseline[32]. In contrast to the effects observed at low concentrations, high concentrations of Na_2S showed clear detrimental effects: no motility or active mitochondria were observed in any sample treated with 300 μM Na_2S. The toxic effects were acute and led to immediate failure of sperm motility and mitochondrial activity (personal observations). This phenomenon can be explained by the high levels of ROS induced by Na_2S[9], an explanation corroborated by the increased levels of lipid peroxidation and the low percentage of sperm with intact plasma membrane and acrosome (NAR) found in our study. In contrast to our findings, in testicular germ cells, Li *et al.* found that treatment with NaHS in the concentration range of 1–200 μM for 30 minutes does not affect cell viability, although cell injuries are induced at 1 mM[4]. This result might be because, unlike other male germ cells, spermatozoa have limited antioxidant defences. Spermatozoa lack the necessary cytoplasmic-enzyme repair systems, and their membranes are particularly rich in polyunsaturated fatty acids, which make these cells particularly susceptible to the damage caused by oxidative stress[16]. Another reason might be related to the use of open systems (e.g. four-well plates and Petri dishes) that lead to quick volatilisation of the gas during cell incubation[33]. It is known that Na_2S volatilises very quickly[33], and the use of closed systems, such as the one used in our study, delays this process and leads to more consistent results regarding the effects of H_2S donors on cell biology. Our results also show that Na_2S and GYY4137 partly reduce the damage induced by high ROS levels on acrosomal status by decreasing the percentage of acrosome-lost (PNA-FITC) spermatozoa relative to that in the CTR-ox group. However, neither of the two donors showed any positive effect on the acrosome integrity evaluated by the NAR test. This divergence could be explained by the different acrosomal attributes considered by these two methodologies: NAR evaluated in unstained samples by phase-contrast microscopy versus outer acrosomal membrane integrity assessed by PNA-FITC staining with epifluorescence microscopy[34,35]. Because acrosome integrity is a requisite for fertilisation and the ROS levels affect sperm function[36,37], it remains to be tested whether Na_2S and GYY4137 may preserve the fertilising potential of sperm cells under oxidative stress. Semen handling and storage decrease boar sperm quality and fertilising ability, probably because of oxidative stress[38,39], so H_2S donors may be useful for the optimisation of semen extenders used in artificial insemination programmes. In the porcine industry, higher efficiency of artificial insemination outcomes may have major economic implications because this assisted reproductive technology is the main tool for pig gene dissemination worldwide[40].

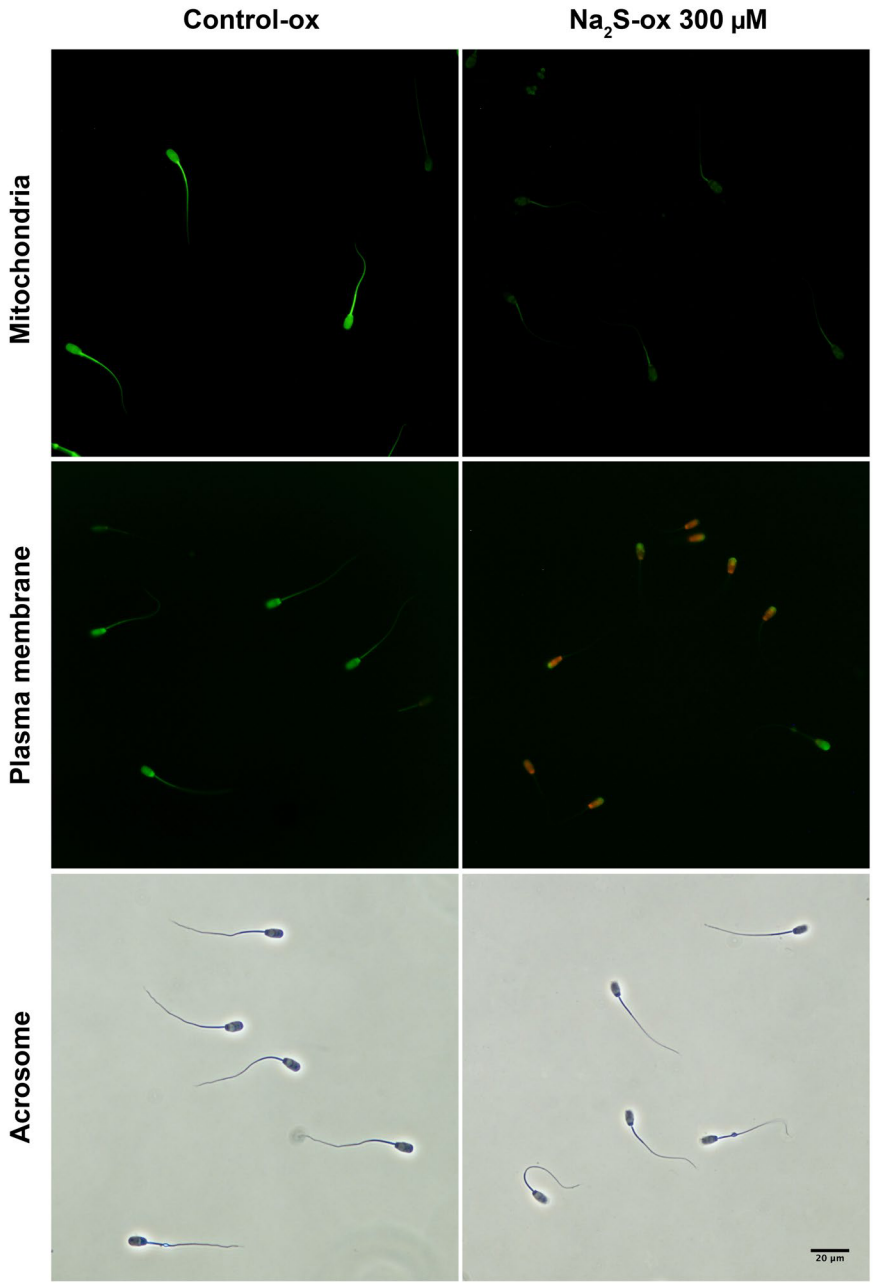


Figure 3. Detrimental effects of a high concentration of Na_2S, a fast-releasing H_2S donor, on the boar sperm mitochondrial status and the plasma membrane and acrosome integrities (normal apical ridge) under oxidative stress. Representative images of sperm cells assessed by epifluorescence microscopy (mitochondrial status was assessed by using rhodamine 123 and propidium iodide; plasma membrane integrity was assessed by using carboxyfluorescein diacetate and propidium iodide) or phase-contrast microscopy (the normal apical ridge was assessed after fixation with glutaraldehyde).

Several mechanisms are involved in the ROS scavenging properties of H_2S. One of these mechanisms involves H_2S itself by virtue of its reducing properties[13]. In this way, the total antioxidant capacities of Na_2S and GYY4137 were tested in this study based on the compounds' abilities to reduce 2,2′-azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid) (ABTS) previously oxidised with H_2O_2[41]. Another mechanism that may explain the capacity of H_2S donors to palliate the damages provoked by oxidative stress concerns the enhancement of the cellular antioxidant defences. Previous studies found that H_2S increases glutathione synthesis, decreases ROS production, and stimulates the activities of superoxide dismutase, glutathione peroxidase, and glutathione reductase[4,9,13,25,26]. Moreover, H_2S decreases the apoptosis rate, increases the protein expression ratio of Bax/Bcl-2, and stimulates Cyp19 gene expression, among other effects[4,18,42]. Because spermatozoa are transcriptionally inactive[43], it is likely that H_2S donors protect sperm cells against ROS damage through their proper reducing activity, as well as by increasing the ratio of reduced to oxidised glutathione and stimulating superoxide

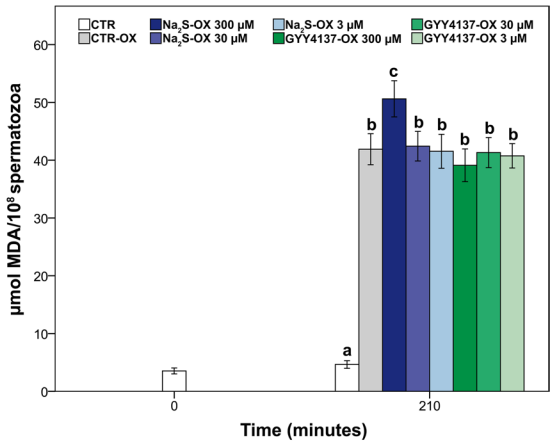


Figure 4. Lipid peroxidation in boar sperm samples submitted to oxidative stress and supplemented with the H_2S donors Na_2S and GYY4137. CTR: control; ox: samples submitted to induced oxidative stress; MDA: malondialdehyde. Different letters indicate significant differences ($p < 0.05$) among treatments. Data are shown as the mean±standard error of six replicates.

dismutase and glutathione peroxidase activities, which represent the major antioxidant defence system of sperm cells[44]. Further studies are nevertheless required to investigate the mechanisms of action of H_2S donors in sperm cells under oxidative stress.

In conclusion, our study provides evidence about the antioxidant properties of two H_2S donors, Na_2S and GYY4137; this evidence will be useful for future studies aiming to test the antioxidant effect of this gasotransmitter. Our findings clearly show that Na_2S has a shorter and less stable total antioxidant capacity than that of GYY4137; it is even undetectable by spectrophotometry at 150 µM. However, it is important to stress out that the antioxidant capacity of GYY4137 tends to increase over time. We also found that both H_2S donors preserve sperm motility and protect the acrosomal status against the detrimental consequences of oxidative stress, although the effects were clearly both dose and donor dependent. Within the range of concentrations tested (3–300 µM), GYY4137 showed positive effects on sperm motility, whereas Na_2S was detrimental at the highest concentration but beneficial at the lowest. Taken together, our results suggest that Na_2S and GYY4137 may be used as *in vitro* therapeutic agents against oxidative stress in sperm cells, although the optimal therapeutic range varies between H_2S donors.

Methods

Reagents were purchased from Sigma–Aldrich (Prague, Czech Republic), unless otherwise indicated.

Ethics statement. This study did not involve animal handling because the sperm samples were purchased as artificial insemination doses from a pig breeding company (Chovservis, Hradec Králové, Czech Republic).

Experiment I. This experiment was designed to evaluate the total antioxidant capacity and stability of Na_2S and GYY4137 at 20, 120, and 210 minutes during incubation at 38 °C in a water bath. The solutions were prepared shortly before the experiment and kept in microcentrifuge tubes tightly sealed with the attached cap (certified free of DNA, DNase, RNase, and endotoxins (pyrogens); material: virgin polypropylene; volume: 600 µl; Neptune Scientific, San Diego, CA, USA) during the whole incubation. For each concentration of H_2S donor, analyses were performed on the same tube throughout the incubation period. Moreover, each microcentrifuge tube contained the same volume (i.e. 200 µl) of H_2S donor or phosphate-buffered saline (PBS; blank) solution. The experiment was replicated four times.

H_2S donor preparation. Na_2S ($Na_2S \times 9\ H_2O$) and GYY4137 ($C_{11}H_{16}NO_2PS_2{\cdot}C_4H_9NO \times CH_2Cl_2$) were prepared in PBS (pH≈7) solution at final concentrations of 2,400, 1,200, 600, 300, and 150 µM.

Total antioxidant capacity of H_2S donors. The total antioxidant capacity was determined by spectrophotometry (Libra S22, Biochrom, Harvard Bioscience Company, Cambourne, United Kingdom) at 660 nm by using the method described previously[41]. The principle of this assay is based on the antioxidant's capacity to reduce ABTS previously oxidised with H_2O_2. A standard curve was established by using known concentrations of 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox). The total antioxidant capacity was expressed as Trolox equivalents (µM or mM). The assay was run in duplicate for each sample.

Experiment II. This experiment was designed to test whether H_2S donors protect sperm cells against the deleterious effects of oxidative stress.

Sample collection and experimental design. Artificial insemination doses from 18 boars of different breeds were purchased from a pig breeding company. Sperm-rich fractions were collected by the gloved-hand method, diluted with Solusem® extender (AIM Worldwide, Vught, Netherlands; pH≈7), and transported to the laboratory at 17 °C.

The sperm motility and morphology were then checked, and only samples with at least 75% of motile and morphologically normal sperm were used for the experiments. Sperm samples from three boars were pooled to reduce the effect of male variability and were centrifuged at 167 *g* for 3 minutes at 17 °C to remove debris and dead sperm cells. The sperm concentration was then checked by using a Bürker chamber, adjusted to 30–40 × 10^6 spermatozoa/ml with Solusem®, and finally diluted 1:1 (v/v) with Solusem® supplemented with 0.2% (w/v) of bovine serum albumin (BSA; ethanol-fractionated lyophilised powder). Thus, the final sperm and BSA concentrations were 15–20 × 10^6 spermatozoa/ml and 0.1%, respectively. Sperm samples were then randomly split into eight microcentrifuge tubes tightly sealed with the attached cap (certified free of DNA, DNase, RNase, and endotoxins (pyrogens); material: virgin polypropylene; volume: 2 ml; Neptune Scientific, San Diego, CA, USA): CTR, CTR-ox, and Na_2S or GYY4137 at 300, 30, and 3 µM under oxidative stress. Oxidative stress was induced by adding a solution composed of 0.1 mM $FeSO_4$ and 0.5 mM sodium ascorbate (Fe^{2+}/ascorbate) to the sperm samples. Because the effects of this ROS-generating system are clearly evident after 210 minutes of sperm incubation[19], sperm analyses were performed at 0 hour (after 20 minutes of incubation for the CTR group only) and after 210 minutes of incubation at 38 °C in a water bath. The experiment was replicated six times with six independent semen pools.

Sperm motility. Sperm motility was evaluated by using CASA (NIS-Elements; Nikon, Tokyo, Japan, and Laboratory Imaging, Prague, Czech Republic), after loading 5 µl of sperm sample into a pre-warmed (38 °C) Spermtrack chamber (PROiSER R + D S.L., Paterna, Spain; chamber depth: 20 µm). A total of ten sperm kinetic parameters were obtained by analysing six random fields: TM (%), PM (%),VAP (µm/s), VCL (µm/s), VSL (µm/s), ALH (µm), BCF (Hz), LIN (VSL/VCL, %), STR (VSL/VAP, %), and WOB (VAP/VCL, %). The settings parameters were as follows: frames per second, 60; minimum frames acquired, 31; VAP ≥ 10 µm/s to classify a spermatozoon as motile, STR ≥ 80% to classify a spermatozoon as progressive[19]. A minimum of 200 sperm cells were analysed for each sample.

Sperm mitochondrial status. Mitochondrial status was evaluated as previously described[45], with minor modifications. Briefly, aliquots of sperm samples were incubated with rhodamine 123 (5 mg/ml, w/v, in dimethyl sulfoxide, DMSO) and propidium iodide (0.5 mg/ml, w/v, in PBS) for 15 minutes at 38 °C in the dark. Subsequently, samples were centrifuged at 500 *g* for 5 minutes, the supernatant was removed, and the sperm pellet was resuspended in PBS. Then, 200 spermatozoa were evaluated by using epifluorescence microscopy (40× objective; Nikon Eclipse E600, Nikon, Tokyo, Japan): the spermatozoa showing bright green fluorescence in the midpiece were considered to have active mitochondria.

Sperm plasma membrane integrity. The sperm plasma membrane integrity was evaluated as previously described[46,47]. Aliquots of sperm samples were incubated with carboxyfluorescein diacetate (0.46 mg/ml, w/v, in DMSO), propidium iodide (0.5 mg/ml, w/v, in PBS), and formaldehyde solution (0.3%, v/v) for 10 minutes at 38 °C in the dark. Then, 200 spermatozoa were evaluated by using epifluorescence microscopy (40× objective). The spermatozoa showing green fluorescence over the entire head area were considered to have intact plasma membrane.

Lipid peroxidation. Lipid peroxidation was assessed with the thiobarbituric acid reactive substances (TBARS) assay, as previously described[19,48]. At the end of each incubation period, sperm aliquots were collected and stored at −80 °C until analysis. The absorbance of each sample was then measured by spectrophotometry at 532 nm. A standard curve was established by using known concentrations of 1,1,3,3-tetramethoxypropane (MDA). The levels of lipid peroxidation are shown as µmol of MDA per 10^8 spermatozoa. The assay was run in duplicate for each sample.

Acrosomal status. Acrosome integrity was assessed after sample fixation in 2% (v/v) glutaraldehyde solution and by examination with phase-contrast microscopy (40× objective)[34]. For each sample, 200 spermatozoa were evaluated, and the percentage of sperm cells with NAR was determined. Acrosome loss was evaluated according to the protocol previously described[49]. Briefly, after methanol fixation and double washing with PBS, the samples were incubated with PNA-FITC (100 µg/ml, w/v, in PBS) for 10 minutes at 38 °C in the dark. Epifluorescence microscopy (40× objective) was used to evaluate 200 spermatozoa, and the cells showing no fluorescence over the acrosome were considered as acrosome-lost spermatozoa.

Statistical analysis. Data were analysed with the statistical program SPSS, version 20 (IBM Inc., Chicago, IL, USA). Shapiro-Wilk's and Levene's tests were used to analyse the normal distribution and the variance homogeneity of the data, respectively. The Mann–Whitney U-test was applied to check for differences between the total antioxidant capacities of Na_2S and GYY4137 at the same concentration, whereas the repeated-measures Friedman test was used to compare the total antioxidant capacities of the H_2S donors across the incubation times. The generalized linear model (GZLM) was performed to analyse the effects of the type and concentration of H_2S donor on the sperm variables. The statistical significance was determined at $p < 0.05$. Data are shown as the mean±standard error.

Data availability

All data generated or analysed during this study are included in this article and its supplementary information file.

Received: 4 November 2019; Accepted: 1 April 2020;
Published online: 16 April 2020

References

1. Kolluru, G. K., Shen, X., Bir, S. C. & Kevil, C. G. Hydrogen sulfide chemical biology: pathophysiological roles and detection. *Nitric Oxide-Biol. Chem.* **35**, 5–20 (2013).
2. Wang, R. Physiological implications of hydrogen sulfide: a whiff exploration that blossomed. *Physiol. Rev.* **92**, 791–896 (2012).
3. Sugiura, Y. *et al*. Cadmium exposure alters metabolomics of sulfur-containing amino acids in rat testes. *Antioxid. Redox Signal.* **7**, 781–787 (2005).
4. Li, G., Xie, Z. Z., Chua, J. M., Wong, P. C. & Bian, J. Hydrogen sulfide protects testicular germ cells against heat-induced injury. *Nitric Oxide-Biol. Chem.* **46**, 165–171 (2015).
5. Gao, D. D. *et al*. Cellular mechanism underlying hydrogen sulfide mediated epithelial K^+ secretion in rat epididymis. *Front. Physiol.* **9**, 1886, https://doi.org/10.3389/fphys.2018.01886 (2019).
6. D'Emmanuele di Villa Bianca, R. *et al*. Hydrogen sulfide as a mediator of human corpus cavernosum smooth-muscle relaxation. *Proc. Natl. Acad. Sci. USA* **106**, 4513–4518 (2009).
7. Martínez-Heredia, J., de Mateo, S., Vidal-Taboada, J. M., Ballescà, J. L. & Oliva, R. Identification of proteomic differences in asthenozoospermic sperm samples. *Hum. Reprod.* **23**, 783–791 (2008).
8. Wang, J. *et al*. Hydrogen sulfide as a potential target in preventing spermatogenic failure and testicular dysfunction. *Antioxid. Redox Signal.* **28**, 1447–1462 (2018).
9. Zhao, Y. *et al*. Hydrogen sulfide and/or ammonia reduces spermatozoa motility through AMPK/AKT related pathways. *Sci. Rep.* **6**, 37884, https://doi.org/10.1038/srep37884 (2016).
10. Song, Z. J. *et al*. Hydrogen sulfide donors in research and drug development. *Med. Chem. Commun.* **5**, 557–570 (2014).
11. Wu, D., Hu, Q. & Zhu, Y. Therapeutic application of hydrogen sulfide donors: the potential and challenges. *Front. Med* **10**, 18–27 (2016).
12. Rose, P., Dymock, B. W. & Moore, P. K. GYY4137, a novel water-soluble, H_2S-releasing molecule in *Methods in Enzymology* (eds. Cadenas, E. & Packer, L.) 143–167 (Elsevier, 2015).
13. Kimura, Y. & Kimura, H. Hydrogen sulfide protects neurons from oxidative stress. *FASEB J.* **18**, 1165–1167 (2004).
14. Yonezawa, D. *et al*. A protective role of hydrogen sulfide against oxidative stress in rat gastric mucosal epithelium. *Toxicology* **241**, 11–18 (2007).
15. Han, W., Dong, Z., Dimitropoulou, C. & Su, Y. Hydrogen sulfide ameliorates tobacco smoke-induced oxidative stress and emphysema in mice. *Antioxid. Redox. Signal.* **15**, 2121–2134 (2011).
16. Agarwal, A., Virk, G., Ong, C. & du Plessis, S. S. Effect of oxidative stress on male reproduction. *World J. Mens Health* **32**, 1–17 (2014).
17. Aitken, R. J., Gibb, Z., Baker, M. A., Drevet, J. & Gharagozloo, P. Causes and consequences of oxidative stress in spermatozoa. *Reprod. Fertil. Dev.* **28**, 1–10 (2016).
18. Ning, J. Z. *et al*. The protective effects of GYY4137 on testicular torsion/detorsion injury in rats. *Int. J. Clin. Exp. Med.* **11**, 3387–3395 (2018).
19. Pintus, E., Kadlec, M., Jovičić, M., Sedmíková, M. & Ros-Santaella, J. L. Aminoguanidine protects boar spermatozoa against the deleterious effects of oxidative stress. *Pharmaceutics* **10**, 212, https://doi.org/10.3390/pharmaceutics10040212 (2018).
20. Martín Muñoz, P. *et al*. Redox cycling induces spermptosis and necrosis in stallion spermatozoa while the hydroxyl radical (OH•) only induces spermptosis. *Reprod. Domest. Anim.* **53**, 54–67 (2018).
21. Barranco, I. *et al*. High total antioxidant capacity of the porcine seminal plasma (SP-TAC) relates to sperm survival and fertility. *Sci. Rep.* **5**, 18538, https://doi.org/10.1038/srep18538 (2015).
22. Cooper, C. E. & Brown, G. C. The inhibition of mitochondrial cytochrome oxidase by the gases carbon monoxide, nitric oxide, hydrogen cyanide and hydrogen sulfide: chemical mechanism and physiological significance. *J. Bioenerg. Biomembr.* **40**, 533–539 (2008).
23. Whiteman, M. *et al*. The effect of hydrogen sulfide donors on lipopolysaccharide-induced formation of inflammatory mediators in macrophages. *Antioxid. Redox Signal.* **12**, 1147–1154 (2010).
24. Kim, T. J., Lee, Y. J., Ahn, Y. J. & Lee, G. J. Characterization of H_2S releasing properties of various H_2S donors utilizing microplate cover-based colorimetric assay. *Anal. Biochem.* **574**, 57–65 (2019).
25. Kimura, Y., Goto, Y. & Kimura, H. Hydrogen sulfide increases glutathione production and suppresses oxidative stress in mitochondria. *Antioxid. Redox Signal.* **12**, 1–13 (2010).
26. Benetti, L. R. *et al*. Hydrogen sulfide inhibits oxidative stress in lungs from allergic mice *in vivo*. *Eur. J. Pharmacol.* **698**, 463–469 (2013).
27. Buettner, G. R. & Jurkiewicz, B. A. Catalytic metals, ascorbate and free radicals: Combinations to avoid. *Radiat. Res.* **145**, 532–541 (1996).
28. Cao, X. *et al*. Renal protective effect of hydrogen sulfide in cisplatin-induced nephrotoxicity. *Antioxid. Redox Signal.* **29**, 455–470 (2018).
29. Szabo, C. *et al*. Regulation of mitochondrial bioenergetic function by hydrogen sulfide. Part I. Biochemical and physiological mechanisms. *Br. J. Pharmacol.* **171**, 2099–2122 (2014).
30. Wiliński, B. *et al*. Sodium hydrosulfide exerts a transitional attenuating effect on spermatozoa migration *in vitro*. *Folia Biol.* **63**, 145–149 (2015).
31. Storey, B. T. Mammalian sperm metabolism: oxygen and sugar, friend and foe. *Int. J. Dev. Biol.* **52**, 427–437 (2008).
32. Elrod, J. W. *et al*. Hydrogen sulfide attenuates myocardial ischemia-reperfusion injury by preservation of mitochondrial function. *Proc. Natl. Acad. Sci. USA* **104**, 15560–15565 (2007).
33. DeLeon, E. R., Stoy, G. F. & Olson, K. R. Passive loss of hydrogen sulfide in biological experiments. *Anal. Biochem.* **421**, 203–207 (2012).
34. Pursel, V. G., Johnson, L. A. & Rampacek, G. B. Acrosome morphology of boar spermatozoa incubated before cold shock. *J. Anim. Sci* **34**, 278–283 (1972).
35. Mortimer, D., Curtis, E. F. & Miller, R. G. Specific labelling by peanut agglutinin of the outer acrosomal membrane of the human spermatozoon. *J. Reprod. Fertil.* **81**, 127–135 (1987).
36. Sancho, S. & Vilagran, I. The boar ejaculate: sperm function and seminal plasma analyses in *Boar Reproduction: Fundamentals and New Biotechnological Trends* (eds. Bonet, S., Casas, I., Holt, W. V. & Yeste, M.) 471–516 (Springer, 2013).
37. Awda, B. J., Mackenzie-Bell, M. & Buhr, M. M. Reactive oxygen species and boar sperm function. *Biol. Reprod* **81**, 553–561 (2009).
38. Kumaresan, A. *et al*. Preservation of boar semen at 18 degrees C induces lipid peroxidation and apoptosis like changes in spermatozoa. *Anim. Reprod. Sci.* **110**, 162–171 (2009).
39. Lopez Rodriguez, A., Van Soom, A., Arsenakis, I. & Maes, D. Boar management and semen handling factors affect the quality of boar extended semen. *Porcine Health Manag.* **3**, 15, https://doi.org/10.1186/s40813-017-0062-5 (2017).
40. Roca, J., Parrilla, I., Bolarin, A., Martinez, E. A. & Rodriguez-Martinez, H. Will AI in pigs become more efficient? *Theriogenology* **86**, 187–196 (2016).
41. Erel, O. A novel automated direct measurement method for total antioxidant capacity using a new generation, more stable ABTS radical cation. *Clin. Biochem.* **37**, 277–285 (2004).
42. Hassan, E., Kahilo, K., Kamal, T., El-Neweshy, M. & Hassan, M. Protective effect of diallyl sulfide against lead-mediated oxidative damage, apoptosis and down-regulation of CYP19 gene expression in rat testes. *Life Sci.* **226**, 193–201 (2019).

43. Grunewald, S., Paasch, U., Glander, H. J. & Anderegg, U. Mature human spermatozoa do not transcribe novel RNA. *Andrologia* **37**, 69–71 (2005).
44. Tvrdá, E., Kňažická, Z., Bárdos, L., Massányi, P. & Lukáč, N. Impact of oxidative stress on male fertility—A review. *Acta Vet. Hung.* **59**, 465–484 (2011).
45. Fraser, L., Lecewicz, M. & Strzezek, J. Fluorometric assessments of viability and mitochondrial status of boar spermatozoa following liquid storage. *Pol. J. Vet. Sci.* **5**, 85–92 (2002).
46. Harrison, R. A. P. & Vickers, S. E. Use of fluorescent probes to assess membrane integrity in mammalian spermatozoa. *J. Reprod. Fertil.* **88**, 343–352 (1990).
47. Grieblová, A., Pintus, E. & Ros-Santaella, J. L. Integrity of head and tail plasmalemma is associated with different kinetic variables in boar sperm. *Anim. Reprod. Sci.* **184**, 218–227 (2017).
48. Brzezińska-Ślebodzińska, E., Ślebodziński, A. B., Pietras, B. & Wieczorek, G. Antioxidant effect of vitamin E and glutathione on lipid peroxidation in boar semen plasma. *Biol. Trace Elem. Res.* **47**, 69–74 (1995).
49. García-Vázquez, F. A. *et al.* Morphometry of boar sperm head and flagellum in semen backflow after insemination. *Theriogenology* **84**, 566–574 (2015).

Acknowledgements

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Author contributions

E.P. and J.L.R.-S. conceived and designed the experiments; E.P., M.J., M.K. and J.L.R.-S. performed the experiments; E.P. and J.L.R.-S. analysed the data; E.P. wrote the paper; E.P., M.J., M.K. and J.L.R.-S. revised and edited the paper.

Competing interests

The authors declare no competing interests.

Additional information

Supplementary information is available for this paper at https://doi.org/10.1038/s41598-020-63489-4.

Correspondence and requests for materials should be addressed to E.P. or J.L.R.-S.

Reprints and permissions information is available at www.nature.com/reprints.

Publisher's note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

pharmaceutics

MDPI

Article

Aminoguanidine Protects Boar Spermatozoa against the Deleterious Effects of Oxidative Stress

Eliana Pintus, Martin Kadlec, Marija Jovičić, Markéta Sedmíková and José Luis Ros-Santaella *

Department of Veterinary Sciences, Faculty of Agrobiology, Food and Natural Resources, Czech University of Life Sciences Prague, Kamýcká 129, 165 00 Praha 6-Suchdol, Czech Republic; eliana.pintus27@gmail.com (E.P.); ma.kadlec13@email.cz (M.K.); jovicic@af.czu.cz (M.J.); sedmikova@af.czu.cz (M.S.)

* Correspondence: rossantaella@gmail.com; Tel.: +420-22438-2932

Received: 6 October 2018; Accepted: 26 October 2018; Published: 1 November 2018

Abstract: Aminoguanidine is a selective inhibitor of the inducible nitric oxide synthase (iNOS) and a scavenger of reactive oxygen species (ROS). Numerous studies have shown the antioxidant properties of aminoguanidine in several cell lines, but the in vitro effects of this compound on spermatozoa under oxidative stress are unknown. In this study, we tested the hypothesis that aminoguanidine may protect against the detrimental effects of oxidative stress in boar spermatozoa. For this purpose, sperm samples were incubated with a ROS generating system (Fe^{2+}/ascorbate) with or without aminoguanidine supplementation (10, 1, and 0.1 mM). Our results show that aminoguanidine has powerful antioxidant capacity and protects boar spermatozoa against the deleterious effects of oxidative stress. After 2 h and 3.5 h of sperm incubation, the samples treated with aminoguanidine showed a significant increase in sperm velocity, plasma membrane and acrosome integrity together with a reduced lipid peroxidation in comparison with control samples ($p < 0.001$). Interestingly, except for the levels of malondialdehyde, the samples treated with 1 mM aminoguanidine did not differ or showed better performance than control samples without Fe^{2+}/ascorbate. The results from this study provide new insights into the application of aminoguanidine as an in vitro therapeutic agent against the detrimental effects of oxidative stress in semen samples.

Keywords: antioxidant capacity; lipid peroxidation; nitric oxide; oxidative stress; sperm velocity

1. Introduction

Oxidative stress arises when the production of the reactive oxygen species (ROS) overwhelms the intrinsic antioxidant defense of a biological system, leading to cell damage and death [1]. As a result of their metabolic activity, cells normally produce ROS, which are also required at certain levels for processes, such as cell signaling, mitochondrial function, and immune response [2–4]. Several factors (e.g., age, cigarette smoke, and ionizing radiation) and pathological conditions (e.g., cancer, diabetes, and infections) can also increase the amount of ROS to be above physiological levels, leading to oxidative stress. Across cells, spermatozoa are particularly susceptible to the damage caused by oxidative stress due to the high content of polyunsaturated fatty acids in their membranes and their limited antioxidant defence [5–7]. Despite certain levels of ROS being required for normal sperm function, their overproduction (due to pathological conditions, semen handling and storage) is detrimental for male fertility both in humans [8,9] and domestic animals [6,10].

Nitric oxide ($NO^{\bullet}$) is a short-living gas and a free radical that participates in many physiological (e.g., immune response, regulation of vascular tone and permeability) and pathological (e.g., cancer and neurological diseases) processes [11–14]. In the male reproductive system, $NO^{\bullet}$ contributes to penile erection, sperm motility, capacitation, hyperactivation, and acrosome reaction [15,16].

In biological systems, $NO^{\bullet}$ can be generated through non-enzymatic pathways by either direct disproportionation or reduction of nitrite under acidic and highly reduced conditions [17]. However, $NO^{\bullet}$ is mainly synthesized from L-arginine by three NO synthase (NOS) isoforms: Neuronal (nNOS), endothelial (eNOS), and inducible (iNOS). All isoforms play a major role in the control of reproductive processes [18] and are expressed in human, mouse, and boar spermatozoa [19–21] among others. Unlike the other isoforms, iNOS is calcium independent and generates a large amount of $NO^{\bullet}$ over prolonged periods (from seconds to days) [18]. Moreover, the iNOS isoform is expressed during inflammation or infection in activated leukocytes [13], which are the main source of ROS in the semen together with abnormal spermatozoa [22,23]. Therefore, the inhibition of the iNOS isoform may contribute by protecting against the detrimental effects of the oxidative stress in the semen.

Aminoguanidine is a selective inhibitor of the iNOS isoform [24] and a scavenger of hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl), hydroxyl ($^{\bullet}OH$) and peroxynitrite ($ONOO^{\bullet}$) radicals [25]. Moreover, aminoguanidine was the first inhibitor of the advanced glycation pathway [26] with similar effects to those of the polyamines, spermine, and spermidine, which are abundant in sperm samples [27]. In a recent study conducted by our research group [28], we found that aminoguanidine improves some sperm kinetic parameters during boar semen storage at 17 °C. Moreover, aminoguanidine protects against the negative effects of oxidative stress induced by environmental pollutants [29] and pathological conditions, such as varicocele [30–33]. Nonetheless, the in vitro effects of aminoguanidine on sperm cells under induced oxidative stress are still unknown.

The aim of this study was to evaluate the in vitro effects of aminoguanidine on sperm cells under induced oxidative stress. Due to its powerful antioxidant activity, we hypothesized that aminoguanidine may protect against the deleterious effects of oxidative stress in sperm samples. To test our hypothesis, sperm samples were treated with Fe^{2+}/ascorbate, which induces lipid peroxidation by catalyzing the production of $^{\bullet}OH$, the most potent free radical known [34]. The total antioxidant capacity, lipid peroxidation, sperm kinetics, plasma membrane integrity, and acrosomal status were evaluated in samples treated with aminoguanidine (10, 1, and 0.1 mM) and compared to those of control samples with or without oxidative stress. The results from this study indicate that aminoguanidine could be used as an efficient in vitro therapeutic agent for the treatment of sperm disorders associated with oxidative stress.

2. Materials and Methods

Reagents were purchased from Sigma-Aldrich (Prague, Czech Republic) unless otherwise stated.

2.1. Collection and Processing of Sperm Samples

Commercial sperm doses from 15 boars of different breeds (i.e., Czech Landrace, Czech Large White, Pietrain, Duroc, and Přeštice Black-Pied) and hybrid genetic lines were purchased from a breeding company (Chovservis, Hradec Králové, Czech Republic). Sperm-rich fractions were collected by the gloved-hand method, diluted with Solusem® extender (AIM Worldwide, Vught, Netherlands), and transported to the laboratory at 17 °C. Only sperm samples with at least 75% motile spermatozoa were used for these experiments. To reduce the effect of male variability, equal volumes of sperm doses from three boars were mixed for each replicate. After this, sperm concentration was checked using a Bürker chamber and samples were further diluted with Solusem® to get a final concentration of 20×10^6 spermatozoa/mL. The samples were then randomly allocated into five groups: Control (CTR), control under oxidative stress (CTR-ox), and three treatments of aminoguanidine under oxidative stress (10, 1, and 0.1 mM, respectively). Aminoguanidine was freshly prepared on the day of the experiment (stock solution: 0.2 M) by dissolving aminoguanidine hydrochloride in phosphate buffered saline (PBS) and diluted with sperm samples to give a final concentration of 10, 1, and 0.1 mM. For CTR-ox samples, an equal volume of PBS solution was added. Oxidative stress was induced by 0.05 mM $FeSO_4$ and 0.5 mM sodium ascorbate (Fe^{2+}/ascorbate), a ROS generating system that is specific for inducing lipid peroxidation [35]. The experiment was replicated five times using five different semen pools.

All sperm analyses were performed at 0 h (after 20 min of incubation, control only), 2 h, and 3.5 h of incubation in a water bath at 38 °C (Supplementary Dataset).

2.2. Assessment of Total Antioxidant Capacity

At the end of each incubation time, 300 µL of each sample was centrifuged at 2000× *g* for 10 min. After this, 150 µL of supernatant was collected and stored at −80 °C until analysis. The total antioxidant capacity was determined by spectrophotometry (Libra S22, Biochrom, Harvard Bioscience Company, Cambridge, UK) at 660 nm using the method described by Erel [36]. A standard curve was established using the known concentrations of 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox). The total antioxidant capacity was expressed as mM Trolox equivalents. This assay was run in duplicate for each sample.

2.3. Assessment of Sperm Motility

A sperm aliquot (5 µL) was loaded into a pre-warmed (38 °C) Spermtrack chamber (PROiSER R + D S.L., Paterna, Spain; chamber depth: 20 µm). Sperm motility was evaluated subjectively by estimating the percentage of motile spermatozoa to the nearest 5% and the quality of movement (QM) using a scale from 0 (lowest: No motility) to 5 (highest: Progressive and vigorous movements). The sperm motility index (SMI) was calculated according to the following formula: [% individual motility + (QM × 20)]/2. Sperm kinetics were assessed by Computer Assisted Sperm Analysis (CASA; NIS-Elements, Nikon, Tokyo, Japan and Laboratory Imaging, Prague, Czech Republic), which consists of an Eclipse E600 tri-ocular phase contrast microscope (Nikon, Tokyo, Japan), equipped with a 10× negative phase-contrast objective (Nikon, Tokyo, Japan), a warming stage set at 38 °C (Tokai Hit, Shizuoka, Japan), and a DMK 23UM021 digital camera (The Imaging Source, Bremen, Germany). A total of nine descriptors of sperm kinetics were recorded after analyzing six random fields: Total motility (TM, %), progressive motility (PM, %), average path velocity (VAP, µm/s), curvilinear velocity (VCL, µm/s), straight-line velocity (VSL, µm/s), amplitude of lateral head displacement (ALH, µm), beat-cross frequency (BCF, Hz), linearity (LIN, %), and straightness (STR, %). The standard parameter settings were as follows: Frames per second, 60; minimum of frames acquired, 31; VAP ≥ 10 µm/s to classify a spermatozoon as motile; and STR ≥ 80% to classify a spermatozoon as progressive. A minimum of 200 motile sperm cells were analyzed per sample.

2.4. Assessment of Lipid Peroxidation

Lipid peroxidation was assessed using the thiobarbituric acid reactive substances (TBARS) assay as previously described [35,37]. At the end of each incubation time, sperm aliquots were collected and stored at −80 °C until analysis. The absorbance of the sample was measured by spectrophotometry at 532 nm. A standard curve was established using the known concentrations of 1,1,3,3-tetramethoxypropane (malondialdehyde, MDA). The levels of lipid peroxidation are shown as µmol of MDA per 10^8 spermatozoa. This assay was run in duplicate for each sample.

2.5. Assessment of Sperm Plasma Membrane Integrity

The assessment of head membrane integrity was performed, as previously described [38,39]. Briefly, sperm samples were incubated with carboxyfluorescein diacetate (stock solution: 0.46 mg/mL in dimethyl sulfoxide), propidium iodide (stock solution: 0.5 mg/mL in PBS), and formaldehyde solution (0.3%) for 10 min at 37 °C in the dark. After this, 200 spermatozoa were evaluated in each sample using epi-fluorescence microscopy (40× objective) and the sperm cells showing complete green fluorescence over the head were considered to have an intact head membrane. The tail membrane integrity was determined using the hypoosmotic swelling test as previously described [39,40]. Briefly, sperm samples were diluted into a pre-warmed hypoosmotic solution (7.35 g/L sodium citrate and 13.51 g/L fructose) and incubated for 30 min at 38 °C. At the end of the incubation, 200 spermatozoa

were evaluated using phase-contrast microscopy (40× objective) and the sperm cells showing swollen tails were considered to have an intact tail membrane.

2.6. Assessment of Acrosomal Status

In order to determine the percentage of sperm cells with a normal apical ridge (NAR) [41], the samples were fixed in 2% glutaraldehyde solution and examined under phase contrast microscopy (40× objective). Two-hundred spermatozoa were evaluated for each sample. The percentage of damaged acrosomes was determined according to the protocol described by García-Vázquez et al. [42]. Briefly, sperm samples were smeared onto glass slides, air-dried, and fixed with methanol for 10 min at room temperature. After this, samples were washed twice with PBS and incubated with peanut agglutinin-fluorescein isothiocyanate (PNA-FITC, stock solution: 0.2 mg/mL in PBS) for 10 min at 37 °C in the dark. Finally, the samples were washed for 5 min with PBS and evaluated under epi-fluorescence microscopy (40× objective). Two-hundred spermatozoa were evaluated and the spermatozoa that showed no fluorescence over the acrosome were considered to be damaged spermatozoa.

2.7. Statistical Analysis

The statistical analyses were performed using the SPSS 20.0 statistical software package (IBM Inc, Chicago, IL, USA). The Shapiro-Wilk test was applied to check for a normal distribution of the data. The repeated measures ANOVA or Friedman tests were used to check for differences in sperm parameters in the control group during the different times of incubation. The generalized linear model (GZLM) was performed to analyze the effects of the treatments and storage times on sperm variables. Data are shown as mean ± standard error (SE). Statistical significance was set at $p < 0.05$.

3. Results

3.1. Total Antioxidant Capacity

As shown in Table 1, the total antioxidant capacity of the CTR samples did not change during the whole incubation ($p > 0.05$). At each incubation time, there were also no differences between CTR and CTR-ox groups ($p > 0.05$). Irrespective of the incubation time, 10 mM aminoguanidine showed greater total antioxidant capacity than CTR-ox group ($p < 0.001$ at both incubation times), while 1 mM aminoguanidine showed greater total antioxidant capacity at 3.5 h of incubation only ($p = 0.031$).

Table 1. Total antioxidant capacity of boar sperm samples submitted to oxidative stress (CTR except) and supplemented with aminoguanidine.

Treatment	Time (h)	Total Antioxidant Capacity (mM)
CTR	0	0.4 ± 0.1 ^{a}
CTR	2	0.1 ± 0.0 ^{a,A}
CTR-ox	2	0.4 ± 0.2 ^{A}
Ag10-ox	2	2.1 ± 0.1 ***
Ag1-ox	2	0.5 ± 0.1
Ag0.1-ox	2	0.4 ± 0.3
CTR	3.5	0.2 ± 0.0 ^{a,A}
CTR-ox	3.5	0.2 ± 0.0 ^{A}
Ag10-ox	3.5	2.2 ± 0.1 ***
Ag1-ox	3.5	0.5 ± 0.1 *
Ag0.1-ox	3.5	0.2 ± 0.1

Total antioxidant capacity is expressed as Trolox equivalents. Different superscript lower-case letters indicate significant differences ($p < 0.05$) among times for the control samples without induced oxidative stress. Different superscript upper-case letters indicate significant differences ($p < 0.05$) within each given time between the control samples with and without induced oxidative stress. The asterisks indicate significant differences (* $p < 0.05$; *** $p \leq 0.001$) within each given time between the treatments and the control samples submitted to induced oxidative stress. CTR = control; ox = samples submitted to induced oxidative stress; Ag10 = 10 mM aminoguanidine; Ag1 = 1 mM aminoguanidine; and Ag0.1 = 0.1 mM aminoguanidine. Data are shown as mean ± standard error of 5 replicates.

Furthermore, after 2 h and 3.5 h of incubation, 10 and 1 mM aminoguanidine showed greater total antioxidant capacity than CTR samples ($p < 0.05$).

3.2. Sperm Motility and Kinetics

At 2 h of incubation, there were no significant differences in any sperm kinetic parameter between CTR and CTR-ox groups ($p > 0.05$, Figure 1 and Table 2). Conversely, at 3.5 h of incubation, sperm kinetic parameters (except for the BCF, LIN, and STR) were negatively affected by this ROS generator ($p < 0.05$, Figure 1 and Table 2).

Overall, our results show that aminoguanidine preserved sperm motility under oxidative stress conditions (Figure 1 and Table 2). At both times of incubation, the TM of samples that were treated with 10 and 1 mM aminoguanidine were significantly greater than those of CTR-ox group ($p < 0.05$) with a two-fold increase at 3.5 h of sperm incubation. Nevertheless, it is important to highlight that despite the great percentage of motile spermatozoa observed during the whole incubation, samples treated with 10 mM aminoguanidine tended to display a relatively non-progressive and circular movement. In this way, at 2 h of incubation, samples treated with 10 mM aminoguanidine showed a greater percentage of motile sperm cells, SMI, TM and VCL, but smaller percentage of PM, BCF, LIN, and STR compared to those of CTR-ox samples ($p < 0.05$). There was also a significant increase in the percentage of motile sperm cells, SMI, and TM at 3.5 h of incubation in samples treated with 0.1 mM aminoguanidine compared to those of CTR-ox samples ($p < 0.05$). While there were no differences in the other kinetic parameters at this aminoguanidine concentration, they tended to be greater than those of CTR-ox group ($p > 0.05$).

Interestingly, irrespective of the incubation time, there was no difference in any sperm kinetic parameter between 1 mM aminoguanidine and CTR samples ($p > 0.05$). Conversely, 10 mM aminoguanidine showed greater TM, VCL, and ALH, but smaller PM, BCF, LIN, and STR than CTR group at 2 h of incubation ($p < 0.05$). Sperm kinetic parameters in samples treated with 0.1 mM aminoguanidine did not differ or were significantly smaller than the CTR group ($p < 0.05$).

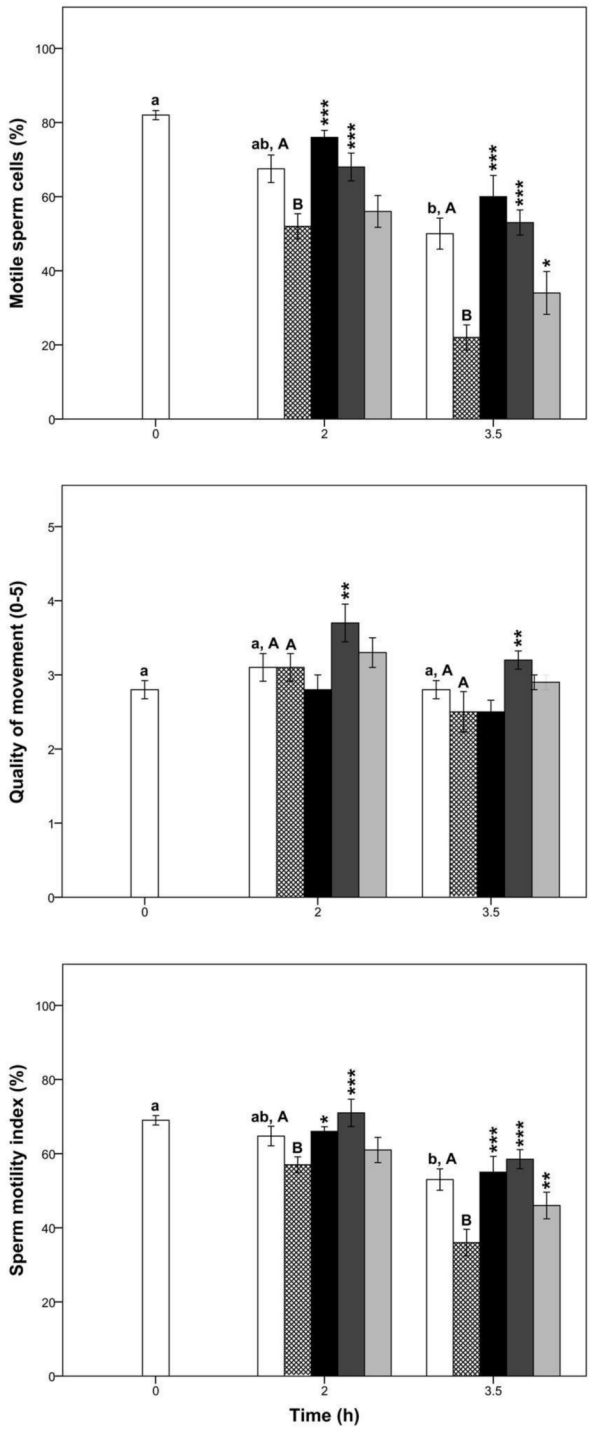


Figure 1. Percentage of motile sperm cells, quality of movement, and sperm motility index in boar samples submitted to oxidative stress (CTR except) and supplemented with aminoguanidine. Different superscript lower-case letters indicate significant differences ($p < 0.05$) among times for the control group without induced oxidative stress. Different superscript upper-case letters indicate significant differences ($p < 0.05$) between the control group with and without induced oxidative stress within each given time. The asterisks indicate significant differences between the treatment and the control submitted to induced oxidative stress within each given time (* $p < 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$). White bars = control samples; crossed bars = control samples under induced oxidative stress; black bars = 10 mM aminoguanidine; dark grey bars = 1 mM aminoguanidine; and light grey bars = 0.1 mM aminoguanidine. Data are shown as mean ± standard error of 5 replicates.

Table 2. Boar sperm kinetics in samples submitted to oxidative stress (CTR except) and supplemented with aminoguanidine.

Treatment	Time (h)	TM (%)	PM (%)	VAP (μm/s)	VCL (μm/s)	VSL (μm/s)	ALH (μm)	BCF (Hz)	LIN (%)	STR (%)
CTR	0	77.2 ± 3.5 ^{a}	44.9 ± 0.9 ^{a}	40.6 ± 1.3 ^{a}	80.8 ± 1.9 ^{a}	30.6 ± 1.0 ^{a}	3.0 ± 0.1 ^{a}	13.1 ± 0.2 ^{a}	38.0 ± 0.9 ^{a}	75.5 ± 0.8 ^{a}
CTR	2	63.1 ± 6.1 ^{a,A}	62.4 ± 5.6 ^{b,A}	39.2 ± 2.8 ^{a,A}	67.5 ± 3.2 ^{b,A}	36.0 ± 2.5 ^{ab,A}	2.7 ± 0.1 ^{b,A}	14.4 ± 0.4 ^{b,A}	51.7 ± 1.4 ^{b,A}	89.7 ± 0.5 ^{b,A}
CTR-ox	2	54.2 ± 4.7 ^{A}	61.2 ± 5.4 ^{A}	41.4 ± 3.0 ^{A}	68.1 ± 5.3 ^{A}	38.9 ± 2.8 ^{A}	2.8 ± 0.2 ^{A}	14.7 ± 0.2 ^{A}	55.9 ± 1.9 ^{A}	92.3 ± 0.9 ^{A}
Ag10-ox	2	83.0 ± 1.6 ***	42.0 ± 2.8 ***	42.2 ± 3.3	92.9 ± 8.6 ***	30.2 ± 1.7 **	3.3 ± 0.3	12.7 ± 0.4 ***	34.2 ± 1.8 ***	70.8 ± 2.3 ***
Ag1-ox	2	65.6 ± 1.4 *	63.2 ± 2.6	45.0 ± 4.9	79.6 ± 9.4	40.7 ± 4.1	3.1 ± 0.4	14.0 ± 0.4	50.8 ± 2.3 *	88.6 ± 1.4 *
Ag0.1-ox	2	56.7 ± 4.7	58.7 ± 3.1	39.4 ± 4.0	68.4 ± 9.0	35.8 ± 3.1	2.7 ± 0.3	14.4 ± 0.4	52.5 ± 3	89.5 ± 2.2
CTR	3.5	60.0 ± 6.3 ^{a,A}	62.8 ± 6.0 ^{b,A}	41.5 ± 3.3 ^{a,A}	68.9 ± 4.2 ^{b,A}	38.6 ± 3.3 ^{b,A}	2.7 ± 0.2 ^{ab,A}	14.8 ± 0.4 ^{b,A}	54.6 ± 1.9 ^{b,A}	90.9 ± 1.0 ^{b,A}
CTR-ox	3.5	24.9 ± 4.4 ^{B}	43.7 ± 8.0 ^{B}	29.4 ± 1.4 ^{B}	45.5 ± 2.9 ^{B}	27.9 ± 1.4 ^{B}	1.9 ± 0.1 ^{B}	15.1 ± 0.4 ^{A}	62.3 ± 2.4 ^{B}	93.6 ± 1.4 ^{A}
Ag10-ox	3.5	69.0 ± 5.6 ***	52.6 ± 4.0	36.0 ± 3.2	78.1 ± 6.6 ***	29.3 ± 2.5	2.8 ± 0.2 ***	12.9 ± 0.4 ***	39.4 ± 1.6 ***	80.5 ± 1.7 ***
Ag1-ox	3.5	59.0 ± 3.8 ***	63.8 ± 3.3 ***	40.4 ± 2.5 **	69.2 ± 3.1 **	37.7 ± 2.5 **	2.8 ± 0.1 **	14.2 ± 0.4	54.1 ± 1.2 ***	91.6 ± 0.6
Ag0.1-ox	3.5	39.6 ± 4.8 *	53.1 ± 5.5	32.7 ± 1.9	53.0 ± 5.0	30.8 ± 1.5	2.3 ± 0.2	15.0 ± 0.4	59.1 ± 2.5	93.0 ± 0.8

Different superscript lower-case letters in the same column indicate significant differences ($p < 0.05$) among times for the control group without induced oxidative stress. Different superscript upper-case letters in the same column indicate significant differences ($p < 0.05$) between the control group with and without induced oxidative stress within each given time. The asterisks indicate significant differences between the treatment and the control submitted to induced oxidative stress within each given time (* $p < 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$). TM = total motility; PM = progressive motility; VAP = average path velocity; VCL = curvilinear velocity; VSL = straight-line velocity; ALH = amplitude of lateral head displacement; BCF = beat-cross frequency; LIN = linearity; STR = straightness; CTR = control; ox = samples submitted to induced oxidative stress; Ag10 = 10 mM aminoguanidine; Ag1 = 1 mM aminoguanidine; and Ag0.1 = 0.1 mM aminoguanidine. Data are shown as mean ± standard error of 5 replicates.

3.3. Lipid Peroxidation

The oxidative stress induced by Fe^{2+}/ascorbate provoked a significant increase in sperm lipid peroxidation at 2 h and 3.5 h of semen incubation ($p < 0.05$; Figure 2) compared to the CTR group. On the other hand, CTR samples did not change their levels of MDA during the entire period of semen incubation ($p > 0.05$). Interestingly, at 2 h of incubation, all aminoguanidine treatments showed lower levels of MDA than those of CTR-ox group ($p < 0.05$). Conversely, at 3.5 h of incubation, only 10 and 1 mM aminoguanidine showed lower levels of lipid peroxidation than CTR-ox samples (both $p < 0.001$).

As expected, all aminoguanidine treatments showed greater levels of lipid peroxidation than CTR samples ($p < 0.001$).

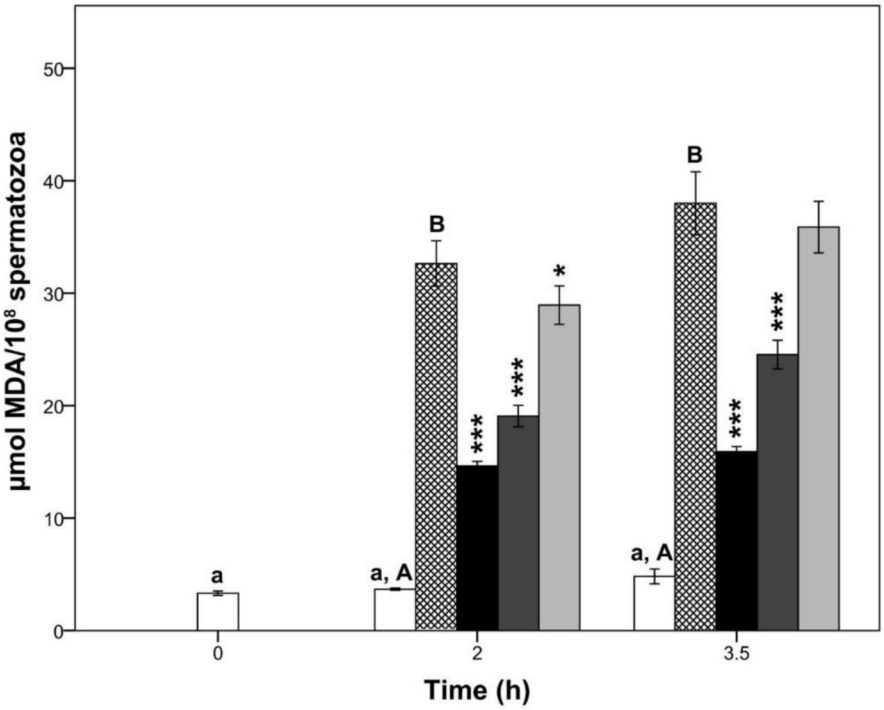


Figure 2. Lipid peroxidation in boar sperm samples submitted to oxidative stress (CTR except) and supplemented with aminoguanidine. Different superscript lower-case letters indicate significant differences ($p < 0.05$) among times for the control group without induced oxidative stress. Different superscript upper-case letters in the same column indicate significant differences ($p < 0.05$) between the control group with and without induced oxidative stress within each given time. The asterisks indicate significant differences between the treatment and the control submitted to induced oxidative stress within each given time (* $p < 0.05$; *** $p \leq 0.001$). MDA = malondialdehyde; white bars = control samples; crossed bars = control samples under induced oxidative stress; black bars = 10 mM aminoguanidine; dark grey bars = 1 mM aminoguanidine; and light grey bars = 0.1 mM aminoguanidine. Data are shown as mean ± standard error of 5 replicates.

3.4. Sperm Plasma Membrane Integrity and Acrosomal Status

In general, the oxidative stress induced by Fe^{2+}/ascorbate impaired the sperm plasma membrane and acrosome integrity of CTR-ox samples (Table 3). Moreover, we found that aminoguanidine protects the sperm head plasma membrane and acrosome integrity against oxidative stress (Table 3). While there were no significant differences in the sperm tail plasma membrane integrity between the values of CTR-ox samples and those of aminoguanidine treatments ($p > 0.05$), there was a trend for the latter to be greater at any concentration used.

Table 3. Boar sperm plasma membrane integrity and acrosomal status in samples submitted to oxidative stress (CTR except) and supplemented with aminoguanidine.

Treatment	Time (h)	Intact Head Plasma Membrane (%)	Intact Tail Plasma Membrane (%)	Normal Apical Ridge (%)	Damaged Acrosome (%)
CTR	0	83.2 ± 0.6 ^{a}	27.9 ± 2.5 ^{a}	94.5 ± 0.3 ^{a}	2.1 ± 0.2 ^{a}
CTR	2	76.4 ± 0.5 ^{b,A}	23.0 ± 4.6 ^{ab,A}	92.1 ± 0.5 ^{b,A}	3.2 ± 0.4 ^{b,A}
CTR-ox	2	71.9 ± 0.7 ^{B}	16.3 ± 4.5 ^{A}	87.6 ± 1.3 ^{B}	3.9 ± 0.4 ^{A}
Ag10-ox	2	78.8 ± 0.6 ***	24.3 ± 4.3	93.1 ± 0.5 ***	2.7 ± 0.3 **
Ag1-ox	2	81.6 ± 1.0 ***	20.2 ± 3.8	91.4 ± 1.1 ***	2.2 ± 0.2 ***
Ag0.1-ox	2	74.3 ± 0.3 *	21.0 ± 4.7	91.1 ± 0.5 ***	3.3 ± 0.4
CTR	3.5	69.3 ± 1.2 ^{c,A}	20.4 ± 4.0 ^{b,A}	88.7 ± 0.8 ^{c,A}	4.7 ± 0.3 ^{c,A}
CTR-ox	3.5	62.8 ± 0.8 ^{B}	14.1 ± 3.7 ^{A}	84.9 ± 1.0 ^{B}	6.2 ± 0.3 ^{B}
Ag10-ox	3.5	73.5 ± 0.8 ***	22.0 ± 3.9	90.3 ± 1.0 ***	4.2 ± 0.4 ***
Ag1-ox	3.5	78.3 ± 1.1 ***	18.1 ± 4.5	90.6 ± 1.1 ***	3.1 ± 0.2 ***
Ag0.1-ox	3.5	67.7 ± 1.2 ***	16.0 ± 4.61	90.5 ± 0.6 ***	5.3 ± 0.3 *

Different superscript lower-case letters in the same column indicate significant differences ($p < 0.05$) among times for the control group without induced oxidative stress. Different superscript upper-case letters in the same column indicate significant differences ($p < 0.05$) between the control group with and without induced oxidative stress within each given time. The asterisks indicate significant differences between the treatment and the control submitted to induced oxidative stress within each given time (* $p < 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$). CTR = control; ox = samples submitted to induced oxidative stress; Ag10 = 10 mM aminoguanidine; Ag1 = 1 mM aminoguanidine; and Ag0.1 = 0.1 mM aminoguanidine. Data are shown as mean ± standard error of 5 replicates.

We also found that 10 and 1 mM aminoguanidine showed a greater percentage of intact sperm head plasma membrane at 2 h ($p = 0.025$ and $p < 0.001$, respectively) and 3.5 h of sperm incubation (both $p < 0.001$) than CTR samples. Moreover, at 2 h and 3.5 h of incubation, 1 mM aminoguanidine showed a lower percentage of damaged acrosome than that of the CTR group ($p = 0.011$ and $p < 0.001$, respectively).

4. Discussion

The present study provides the first piece of evidence that aminoguanidine notably reduces the detrimental effects of oxidative stress in boar sperm cells in vitro. Our results clearly show that aminoguanidine has powerful antioxidant capacity, preserves the sperm motility, reduces the lipid peroxidation, and protects the plasma membrane and acrosome integrity under induced oxidative stress. Interestingly, except for the levels of MDA, sperm parameters of samples treated with 1 mM aminoguanidine did not differ or even showed better performance than those of control samples without the ROS-generating system, which demonstrates that the deleterious effects of oxidative stress were mostly abolished. As there was no cytotoxic effect shown in any sperm parameter, our results suggest that aminoguanidine could potentially be a treatment for impaired semen quality associated with high ROS levels.

Aminoguanidine is a selective inhibitor of the iNOS isoform [24], which releases large amounts of $NO^{\bullet}$ and is found in spermatozoa and activated leukocytes [18,21]. In human semen, the presence of abnormal spermatozoa and activated leukocytes increases the amount of ROS over physiological levels, which causes sperm DNA damage, lipid peroxidation, and poor motility [5]. In this sense, Balercia et al. [43] found that asthenozospermic men show greater levels of $NO^{\bullet}$ than normozoospermic men and that the concentration of this gasotransmitter were negatively correlated with the sperm motility. In this way, our findings indicate that aminoguanidine can be employed for protecting against the effects of oxidative stress in sperm cells, which is consistent with the findings in other cells and tissues (lung: [44]; bladder: [45]; kidney: [46]; testis: [29]). Similarly, Abbasi et al. [30,31] and Alizadeh et al. [32,33] found that the in vivo administration of aminoguanidine improves the sperm concentration, motility, viability, normal morphology, mitochondrial membrane potential,

and DNA integrity in varicocelized rats where the upregulation of the iNOS isoform may lead to high levels of ROS in the semen.

As previously described in boar semen [35,47], our results confirm that Fe^{2+}/ascorbate induces a state of oxidative stress characterized by increased levels of lipid peroxidation and reduced sperm motility. In addition, we also found that this ROS generator negatively affects the sperm head plasma membrane and acrosome integrity. In contrast to our findings, Guthrie and Welch [47] found that Fe^{2+}/ascorbate did not affect the sperm membrane integrity (i.e., viability). This is possibly because a smaller Fe^{2+}/ascorbate concentration was employed (i.e., 1 μM/30 μM) in their study. Our findings also confirm that aminoguanidine has powerful antioxidant abilities against the oxidative stress induced by Fe^{2+}/ascorbate, as previously described by Yildiz et al. [25]. Irrespective of the incubation time, 10 and 1 mM aminoguanidine showed stronger antioxidant capacity than that of control samples with or without induced oxidative stress. The total antioxidant capacity of 1 mM aminoguanidine was 0.5 mM Trolox equivalents on average, which is within the range described in the boar seminal plasma [48,49]. A greater total antioxidant capacity of the seminal plasma contributes to the ability of boar sperm cells to better sustain the preservation process (liquid-storage and cryopreservation), which is also positively related to the fertility outcomes and litter size [49]. In this way, our results indicate that 1 mM aminoguanidine shows a total antioxidant capacity similar to that of boar seminal plasma, which provides further support for the beneficial effects of this compound on boar sperm parameters under induced oxidative stress.

The results of this study show that under induced oxidative stress, aminoguanidine better preserves sperm motility, plasma membrane and acrosome integrity. These three parameters are correlated with male fertility in humans [50] and other species (bulls: [51]; boars: [52]; stallions: [53]). Interestingly, at 3.5 h of incubation, 10 and 1 mM aminoguanidine showed more than twice the percentage of motile sperm cells compared to control samples under oxidative stress. Nevertheless, it is important to highlight that sperm cells treated with 10 mM aminoguanidine showed rapid curvilinear trajectories with remarkably low values of progressive and linear motility. A plausible explanation of this phenomenon might be due to the antioxidant capacity of 10 mM aminoguanidine (2 mM Trolox equivalents), which is quite above the physiological range reported in the boar seminal plasma [48,49]. As certain levels of ROS are required for a normal sperm function [8,9], 10 mM aminoguanidine may reduce the amount of ROS in such a way that it impairs some sperm kinetic parameters, but it does not affect the sperm plasma membrane and acrosome integrity. In this sense, the protective effects on the sperm plasma membrane and acrosome integrity in samples treated with 10 mM aminoguanidine were also confirmed by the lower levels of lipid peroxidation. On the other hand, 0.1 mM aminoguanidine was able to only partially prevent the damage caused by Fe^{2+}/ascorbate in terms of sperm parameters. In contrast, although the sperm tail integrity tended to be greater in sperm samples supplemented with aminoguanidine, there were no differences among the latter and control group treated with Fe^{2+}/ascorbate, which is likely due to the variability among replicates. The boar sperm plasma membrane shows low tolerance to the hypoosmotic conditions, which varies across breeds and between boars within the same breed [54]. In this way, despite the fact that we used pooled semen in order to reduce the male variability, factors, such as the boar and breed, may have influenced our results by increasing the variability among replicates.

Another important finding of this study is that aminoguanidine protects the acrosome integrity as shown by the two techniques employed. It is well known that acrosome integrity is a requisite for the acrosome reaction, which must occur in a timely manner in order to allow the penetration of the spermatozoon through the protective barriers of the oocyte [55]. In the porcine species, a partially induced acrosome reaction in the preincubation or fertilization media has been found to be an important cause of polyspermy, which is one major challenge in the assisted reproductive technologies of this species [56]. The exposure of boar spermatozoa to a ROS generating system triggers the acrosome reaction [57], which may lead to reduced fertilizing potential. Based on our findings, we can therefore speculate that aminoguanidine may increase the fertilization potential of porcine spermatozoa by

preventing a precocious acrosome reaction under oxidative stress. Further studies, such as the in vitro and in vivo fertilizations, are needed to test our hypothesis.

In conclusion, the findings from this study demonstrate that aminoguanidine mostly abolishes the deleterious effects of oxidative stress in boar spermatozoa under in vitro conditions. Due to its antioxidant capacities, aminoguanidine preserves the boar sperm motility, reduces the lipid peroxidation, and protects the plasma membrane and acrosome integrity under oxidative stress. Interestingly, 1 mM aminoguanidine mostly eliminates the negative effects of oxidative stress as, except for the lipid peroxidation, all sperm parameters did not differ or even showed better performance than those of control samples without the ROS-generating system. As no cytotoxic effects were observed in any sperm parameters, our results suggest that aminoguanidine could be used as an effective in vitro therapeutic agent for the treatment of sperm disorders associated with oxidative stress.

Supplementary Materials: The following are available online at http://www.mdpi.com/1999-4923/10/4/212/s1, Supplementary Dataset 1. Dataset of boar sperm parameters under induced oxidative stress (CTR except) and supplemented with aminoguanidine.

Availability of Materials and Data: All data generated or analyzed during this study are included in this article and Supplementary Information file.

Author Contributions: E.P. and J.L.R.S. conceived and designed the experiments; E.P., M.K., M.J., and J.L.R.S. performed the experiments; E.P. and J.L.R.S. analyzed the data; M.S. contributed reagents/materials/analysis tools; E.P. wrote the paper; E.P., M.K., M.J., M.S., and J.L.R.S. revised and edited the paper.

Funding: This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Conflicts of Interest: The authors declare no competing interests.

References

1. Betteridge, D.J. What is oxidative stress? *Metabolism* **2000**, *49*, 3–8. [CrossRef]
2. Giulivi, C.; Poderoso, J.J.; Boveris, A. Production of nitric oxide by mitochondria. *J. Biol. Chem.* **1998**, *273*, 11038–11043. [CrossRef] [PubMed]
3. Birben, E.; Sahiner, U.M.; Sackesen, C.; Erzurum, S.; Kalayci, O. Oxidative stress and antioxidant defense. *World Allergy Organ. J.* **2012**, *5*, 9–19. [CrossRef] [PubMed]
4. Ray, P.D.; Huang, B.W.; Tsuji, Y. Reactive oxygen species (ROS) homeostasis and redox regulation in cellular signaling. *Cell. Signal.* **2012**, *24*, 981–990. [CrossRef] [PubMed]
5. Agarwal, A.; Saleh, R.A. Role of oxidants in male infertility: Rationale, significance, and treatment. *Urol. Clin. N. Am.* **2002**, *29*, 817–827. [CrossRef]
6. Tvrdá, E.; Kňažická, Z.; Bárdos, L.; Massányi, P.; Lukáč, N. Impact of oxidative stress on male fertility—A review. *Acta Vet. Hung.* **2011**, *59*, 465–484. [CrossRef] [PubMed]
7. Aitken, R.J.; Gibb, Z.; Baker, M.A.; Drevet, J.; Gharagozloo, P. Causes and consequences on oxidative stress in spermatozoa. *Reprod. Fertil. Dev.* **2016**, *28*, 1–10. [CrossRef] [PubMed]
8. Agarwal, A.; Virk, G.; Ong, C.; du Plessis, S.S. Effect of oxidative stress on male reproduction. *World J. Mens Health.* **2014**, *32*, 1–17. [CrossRef] [PubMed]
9. Aitken, R.J. Reactive oxygen species as mediators of sperm capacitation and pathological damage. *Mol. Reprod. Dev.* **2017**, *84*, 1039–1052. [CrossRef] [PubMed]
10. Guthrie, H.D.; Welch, G.R. Effects of reactive oxygen species on sperm function. *Theriogenology* **2012**, *78*, 1700–1708. [CrossRef] [PubMed]
11. Knott, A.B.; Bossy-Wetzel, E. Nitric oxide in health and disease of the nervous system. *Antioxid. Redox Signal.* **2009**, *11*, 541–553. [CrossRef] [PubMed]
12. Salimian Rizi, B.; Achreja, A.; Nagrath, D. Nitric oxide: The forgotten child of tumor metabolism. *Trends Cancer.* **2017**, *3*, 659–672. [CrossRef] [PubMed]
13. García-Ortiz, A.; Serrador, J.M. Nitric oxide signaling in T cell-mediated immunity. *Trends Mol. Med.* **2018**, *24*, 412–427. [CrossRef] [PubMed]
14. Helms, C.C.; Gladwin, M.T.; Kim-Shapiro, D.B. Erythrocytes and vascular function: Oxygen and nitric oxide. *Front. Physiol.* **2018**, *9*, 1–9. [CrossRef] [PubMed]

15. Burnett, A.L. Nitric oxide regulation of penile erection: Biology and therapeutic implications. *J. Androl.* **2002**, *23*, S20–S26. [CrossRef] [PubMed]
16. Herrero, M.; Lamirande, E.; Gagnon, C. Nitric oxide is a signaling molecule in spermatozoa. *Curr. Pharm. Des.* **2003**, *9*, 419–425. [CrossRef] [PubMed]
17. Zweier, J.L.; Samouilov, A.; Kuppusamy, P. Non-enzymatic nitric oxide synthesis in biological systems. *Biochim. Biophys. Acta.* **1999**, *1411*, 250–262. [CrossRef]
18. Dixit, V.D.; Parvizi, N. Nitric oxide and the control of reproduction. *Anim. Reprod. Sci.* **2001**, *65*, 1–16. [CrossRef]
19. Herrero, M.B.; Pérez Martínez, S.; Viggiano, J.M.; Polak, J.M.; de Gimeno, M.F. Localization by indirect immunofluorescence of nitric oxide synthase in mouse and human spermatozoa. *Reprod. Fertil. Dev.* **1996**, *8*, 931–934. [CrossRef] [PubMed]
20. Herrero, M.B.; Goin, J.C.; Boquet, M.; Canteros, M.G.; Franchi, A.M.; Perez Martinez, S.; Polak, J.M.; Viggiano, J.M.; Gimeno, M.A. The nitric oxide synthase of mouse spermatozoa. *FEBS Lett.* **1997**, *411*, 39–42. [CrossRef]
21. 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. [CrossRef] [PubMed]
22. Kessopoulou, E.; Tomlinson, M.J.; Barratt, C.L.; Bolton, A.E.; Cooke, I.D. Origin of reactive oxygen species in human semen: Spermatozoa or leucocytes? *J. Reprod. Fertil.* **1992**, *94*, 963–970. [CrossRef]
23. Gomez, E.; Buckingham, D.W.; Brindle, J.; Lanzafame, F.; Irvine, D.S.; Aitken, R.J. Development of an image analysis system to monitor the retention of residual cytoplasm by human spermatozoa: Correlation with biochemical markers of the cytoplasmic space, oxidative stress, and sperm function. *J. Androl.* **1996**, *17*, 276–287. [PubMed]
24. Misko, T.P.; Moore, W.M.; Kasten, T.P.; Nickols, G.A.; Corbett, J.A.; Tilton, R.G.; McDaniel, M.L.; Williamson, J.R.; Currie, M.G. Selective inhibition of the inducible nitric oxide synthase by aminoguanidine. *Eur. J. Pharmacol.* **1993**, *233*, 119–125. [CrossRef]
25. Yildiz, G.; Demiryurek, A.T.; Sahin-Erdemli, I.; Kanzik, I. Comparison of antioxidant activities of aminoguanidine, methylguanidine and guanidine by lumino-enhanced chemiluminescence. *Br. J. Pharmacol.* **1998**, *124*, 905–910. [CrossRef] [PubMed]
26. Borg, D.J.; Forbes, J.M. Targeting advanced glycation with pharmaceutical agents: Where are we now? *Glycoconj. J.* **2016**, *33*, 653–670. [CrossRef] [PubMed]
27. Gugliucci, A.; Menini, T. The polyamines spermine and spermidine protect proteins from structural and functional damage by AGE precursors: A new role for old molecules? *Life Sci.* **2003**, *72*, 2603–2616. [CrossRef]
28. Jovičić, M.; Pintus, E.; Fenclová, T.; Šimonik, O.; Chmelíková, E.; Ros-Santaella, J.L.; Sedmíková, M. Effect of nitric oxide on boar sperm motility, membrane integrity, and acrosomal status during semen storage. *Pol. J. Vet. Sci.* **2018**, *21*, 73–82. [CrossRef] [PubMed]
29. Oguz, F.; Ciftci, O.; Aydın, M.; Timurkaan, N.; Beytur, A.; Altıntas, R.; Parlakpinar, H. Aminoguanidine prevents testicular damage-induced-2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) in male rats. *Andrologia* **2013**, *45*, 225–231. [CrossRef] [PubMed]
30. Abbasi, M.; Alizadeh, R.; Abolhassani, F.; Amidi, F.; Hassanzadeh, G.; Ejtemaei Mehr, S.; Dehpour, A.R. Aminoguanidine improves epididymal sperm parameters in varicocelized rats. *Urol. Int.* **2011**, *86*, 302–306. [CrossRef] [PubMed]
31. Abbasi, M.; Alizadeh, R.; Abolhassani, F.; Amidi, F.; Ragerdi, K.I.; Fazelipour, S.; Hoshino, Y.; Sato, E.; Dehpour, A.R. Effect of aminoguanidine in sperm DNA fragmentation in varicocelized rats: Role of nitric oxide. *Reprod. Sci.* **2011**, *18*, 545–550. [CrossRef] [PubMed]
32. Alizadeh, N.; Abbasi, M.; Abolhassani, F.; Amidi, F.; Mahmoudi, R.; Hoshino, Y.; Sato, E.; Ragerdikashani, I. Effects of aminoguanidine on infertile varicocelized rats: A functional and morphological study. *Daru J. Fac. Pharm.* **2010**, *18*, 51–56.
33. Alizadeh, R.; Navid, S.; Abbasi, N.; Yari, A.; Mazaheri, Z.; Daneshi, E.; Agarwal, A.; Abbasi, M. The effect of aminoguanidine on sperm motility and mitochondrial membrane potential in varicocelized rats. *Iran. J. Basic Med. Sci.* **2016**, *19*, 1279–1284. [CrossRef] [PubMed]
34. Buettner, G.R.; Jurkiewicz, B.A. Catalytic metals, ascorbate and free radicals: Combinations to avoid. *Radiat. Res.* **1996**, *145*, 532–541. [CrossRef] [PubMed]

35. Brzezińska-Ślebodzińska, E.; Ślebodziński, A.B.; Pietras, B.; Wieczorek, G. Antioxidant effect of vitamin E and glutathione on lipid peroxidation in boar semen plasma. *Biol. Trace Elem. Res.* **1995**, *47*, 69–74. [CrossRef]
36. Erel, O. A novel automated direct measurement method for total antioxidant capacity using a new generation, more stable ABTS radical cation. *Clin. Biochem.* **2004**, *37*, 277–285. [CrossRef] [PubMed]
37. Domínguez-Rebolledo, Á.E.; Martínez-Pastor, F.; Fernández-Santos, M.R.; Del Olmo, E.; Bisbal, A.; Ros-Santaella, J.L.; Garde, J.J. Comparison of the TBARS assay and BODIPY C11 probes for assessing lipid peroxidation in red deer spermatozoa. *Reprod. Domest. Anim.* **2010**, *45*, e360–e368. [CrossRef] [PubMed]
38. Harrison, R.A.P.; Vickers, S.E. Use of fluorescent probes to assess membrane integrity in mammalian spermatozoa. *J. Reprod. Fertil.* **1990**, *88*, 343–352. [CrossRef] [PubMed]
39. Grieblová, A.; Pintus, E.; Ros-Santaella, J.L. Integrity of head and tail plasmalemma is associated with different kinetic variables in boar sperm. *Anim. Reprod. Sci.* **2017**, *184*, 218–227. [CrossRef] [PubMed]
40. Jeyendran, R.S.; Van der Ven, H.H.; Perez-Pelaez, M.; Crabo, B.G.; Zaneveld, L.J. Development of an assay to assess the functional integrity of the human sperm membrane and its relationship to other semen characteristics. *J. Reprod. Fertil.* **1984**, *70*, 219–228. [CrossRef] [PubMed]
41. Pursel, V.G.; Johnson, L.A.; Rampacek, G.B. Acrosome morphology of boar spermatozoa incubated before cold shock. *J. Anim. Sci.* **1972**, *34*, 278–283. [CrossRef] [PubMed]
42. García-Vázquez, F.A.; Hernández-Caravaca, I.; Yánez-Quintana, W.; Matás, C.; Soriano-Úbeda, C.; Izquierdo-Rico, M.J. Morphometry of boar sperm head and flagellum in semen backflow after insemination. *Theriogenology* **2015**, *84*, 566–574. [CrossRef] [PubMed]
43. Balercia, G.; Moretti, S.; Vignini, A.; Magagnini, M.; Mantero, F.; Boscaro, M.; Ricciardo-Lamonica, G.; Mazzanti, L. Role of nitric oxide concentrations on human sperm motility. *J. Androl.* **2004**, *25*, 245–249. [CrossRef] [PubMed]
44. Eroglu, C.; Yildiz, O.G.; Saraymen, R.; Soyuer, S.; Kilic, E.; Ozcan, S. Aminoguanidine ameliorates radiation-induced oxidative lung damage in rats. *Clin. Investig. Med.* **2008**, *31*, 182–188. [CrossRef]
45. Abraham, P.; Rabi, S.; Selvakumar, D. Protective effect of aminoguanidine against oxidative stress and bladder injury in cyclophosphamide-induced hemorrhagic cystitis in rat. *Cell Biochem. Funct.* **2009**, *27*, 56–62. [CrossRef] [PubMed]
46. Abo-Salem, O.M. The protective effect of aminoguanidine on doxorubicin-induced nephropathy in rats. *J. Biochem. Mol. Toxicol.* **2012**, *26*, 1–9. [CrossRef] [PubMed]
47. Guthrie, H.D.; Welch, G.R. Use of fluorescence-activated flow cytometry to determine membrane lipid peroxidation during hypothermic liquid storage and freeze-thawing of viable boar sperm loaded with 4, 4-difluoro-5-(4-phenyl-1,3-butadienyl)-4-bora-3a,4a-diaza- s-indacene-3-undecanoic acid. *J. Anim. Sci.* **2007**, *85*, 1402–1411. [CrossRef] [PubMed]
48. Zakošek Pipan, M.; Mrkun, J.; Kosec, M.; Nemec Svete, A.; Zrimšek, P. Superoxide dismutase: A predicting factor for boar semen characteristics for short-term preservation. *Biomed Res. Int.* **2014**, 105280. [CrossRef] [PubMed]
49. Barranco, I.; Tvarijonaviciute, A.; Perez-Patinõ, C.; Parrilla, I.; Ceron, J.J.; Martinez, E.A.; Rodriguez-Martinez, H.; Roca, J. High total antioxidant capacity of the porcine seminal plasma (SP-TAC) relates to sperm survival and fertility. *Sci. Rep.* **2015**, *5*, 1–9. [CrossRef] [PubMed]
50. Khatun, A.; Rahman, M.S.; Pang, M.G. Clinical assessment of the male fertility. *Obstet. Gynecol. Sci.* **2018**, *61*, 179–191. [CrossRef] [PubMed]
51. Kastelic, J.P.; Thundathil, J.C. Breeding soundness evaluation and semen analysis for predicting bull fertility. *Reprod. Domest. Anim.* **2008**, *43*, 368–373. [CrossRef] [PubMed]
52. Jung, M.; Rüdiger, K.; Schulze, M. In vitro measures for assessing boar semen fertility. *Reprod. Domest. Anim.* **2015**, *50*, 20–24. [CrossRef] [PubMed]
53. Love, C.C. Modern techniques for semen evaluation. *Vet. Clin. North Am. Equine Pract.* **2016**, *32*, 531–546. [CrossRef] [PubMed]
54. Druart, X.; Gatti, J.L.; Huet, S.; Dacheux, J.L.; Humblot, P. Hypotonic resistance of boar spermatozoa: Sperm subpopulations and relationship with epididymal maturation and fertility. *Reproduction* **2009**, *137*, 205–213. [CrossRef] [PubMed]
55. Yeste, M. Boar spermatozoa within the oviductal environment (II): Sperm capacitation. In *Boar Reproduction: Fundamentals and New Biotechnological Trends*; Bonet, S., Casas, I., Holt, W.V., Yeste, M., Eds.; Springer: Heidelberg, Germany, 2013; pp. 347–405.

56. Funahashi, H. Polyspermic penetration in porcine IVM-IVF systems. *Reprod. Fertil. Dev.* **2003**, *15*, 167–177. [CrossRef] [PubMed]
57. Awda, B.J.; Mackenzie-Bell, M.; Buhr, M.M. Reactive oxygen species and boar sperm function. *Biol. Reprod.* **2009**, *81*, 553–561. [CrossRef] [PubMed]