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Vliv donoru sulfanu a česnekových derivátů na meiotické zrání a stárnutí prasečích oocytů

doktorská disertační práce

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PROHLÁŠENÍ

Prohlašuji, že jsem doktorskou disertační práci na téma „Vliv donoru sulfanu a česnekových derivátů na meiotické zrání a stárnutí prasečích oocytů“ vypracovala samostatně a použila jen pramenů, které cituji a uvádím v seznamu použité literatury.

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podpis autora

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SEZNAM ZKRATEK

3-MPST	3-mercaptopyruvate-sulfurtransferase	3-merkaptopyruvát sulfurtransferáza
AITI	Anaphase I to telophase I transition	Přechod z anafáze I do telofáze I
AGE	Aged garlic extract	Vyzrálý česnekový extrakt
ATP	Adenosine triphosphate	Adenosintrifosfát
ATPáza	Adenosinetriphosphatase	Adenosintrifosfatáza
cAMP	Cyclic adenosine monophosphate	Cyklický adenosin monofosfát
CBS	Cystathionine-beta-synthase	Cystathionin-beta-syntáza
Ca^{2+}	Calcium ions	Vápenaté ionty
Cdk 1	Cyclin-dependent protein kinase 1	Cyklin-dependentní protein kináza 1
cGMP	Cyclic guanosine monophosphate	Cyklický guanosin monofosfát
COC	Cumulus-oocyte complex	Kumulo-oocytární komplex
CSE	Cystathionine-gamma-lyase	Cystathionin-gama-lyáza
CSF	Cytostatic factor	Cytostatický faktor
DADS	Diallyl disulfide	Diallyl disulfid
DAS	Diallyl sulfide	Diallyl sulfid
DATS	Diallyl trisulfide	Diallyl trisulfid
FSH	Follicle stimulating hormone	Folikuly stimulující hormon
GAG	Glycosaminoglycan	Glykosaminoglykan
GV	Germinal vesicle	Zárodečný váček
GVBD	Germinal vesicle breakdown	Rozpad zárodečného váčku
H1	Histone H1	Histon H1
HA	Hyaluronic acid	Kyselina hyaluronová
JNK	c-Jun-N-terminal kinase	c-Jun-N-terminální kináza
K^{+}	Potassium ions	Draselné ionty

KA	Beta-cyano-L-alanine	Beta-kyano-L-alanin
KGA	Alpha-Ketoglutaric acid	Kyselina alfa-ketoglutarová
LD	Late diakinesis	Pozdní diakineze
LDL	Low density lipoproteins	Lipoproteiny o nízké hustotě
LH	Luteinizing hormone	Luteinizační hormon
MI	Metaphase of the first meiotic division	Metafáze prvního meiotického dělení
MII	Metaphase of the second meiotic division	Metafáze druhého meiotického dělení
MAPK	Mitogen-activated protein kinase	Mitogenem aktivovaná protein kináza
MBP	Myelin basic protein	Myelinový bazický protein
MPF	M-phase promoting factor	M-fázi podporující faktor
Na^+	Sodium ions	Sodné ionty
NADPH	Nicotinamide adenine dinucleotide phosphate	Nikotinamidadenindinukleotidfosfát
NMDA	N-methyl-D-aspartate	N-metyl-D-asparát
OA	Oxamic acid	Kyselina oxamová
OOX	Oocytectomised complex	Oocytektomovaný komplex
PGC	Primordial germ cell	Primordiální zárodečná buňka
PLCζ	Phospholipase C zeta	Fosfolipáza C zeta
ROS	Reactive oxygen species	Reaktivní formy kyslíku
SAC	S-allyl cysteine	S-allyl cystein

Obsah

1 Úvod

Reprodukční biotechnologie jsou významným, rychle se rozvíjejícím odvětvím humánní i veterinární medicíny. Jejich rozvoj závisí na zisku dostatečného množství kvalitních oocytů ve stádiu metafáze II. Meiotické zrání oocytu představuje proces přeměny plně dorostlého oocytu ve stádiu zárodečného váčku v oplození schopný oocyt ve stádiu metafáze II. Proběhne-li proces meiotického zrání úspěšně, je zralý oocyt vývojově kompetentní, tedy schopný vyvíjet se po oplození v životaschopné embryo.

Pokud jsou oocyty po dokončení zrání vystaveny prodloužené kultivaci, nastává proces označovaný jako stárnutí oocytu. Během prodloužené kultivace dochází k poklesu kvality oocytů a k výskytu nežádoucích jevů jako jsou spontánní partenogenetická aktivace, fragmentace a lýza. Oocyty vystavené prodloužené kultivaci vykazují sníženou vnímavost vůči aktivačním stimulům a narušený časný embryonální vývoj po oplození nebo partenogenetické aktivaci.

Dobře nastavený systém pro *in vitro* kultivace oocytů je klíčový pro rozvoj reprodukčních biotechnologií. Kvalitu oocytů ohrožuje řada faktorů, jako jsou změny pH, vystavení přechodnému osvětlení, manuální manipulace a vystavení zvýšeným hladinám kyslíku. Přidáním vhodných sloučenin do kultivačních médií lze pozitivně ovlivnit kvalitu oocytů.

Sulfan patří, spolu s oxidem dusnatým a oxidem uhelnatým, do rodiny gasotransmiterů. Gasotransmitery jsou plynné molekuly, které jsou v organismu endogenně produkovány a plní zde signální funkci. Sulfan je v organismu produkován aktivitou enzymů cystathionin-beta-syntázy, cystathionin-gama-lyázy a 3-merkaptopyruvát-sulfurtransferázy. Přítomnost enzymů produkujících sulfan byla prokázána například v děloze, placentě a folikulárních buňkách.

Česnekové deriváty jsou sirné sloučeniny schopné uvolňovat v organismu sulfan. Česnekové deriváty vykazují v organismu řadu pozitivních účinků; stimulují imunitní systém, mají antimikrobiální, antioxidační a antiproliferativní účinky.

Lze předpokládat, že kvalitu oocytů v *in vitro* podmínkách lze pozitivně ovlivnit přidáním sulfanu a česnekových derivátů do kultivačního média.

2 Literární přehled

2.1 Oogeneze savců

Fáze množení

Oogeneze je proces přeměny primordiálních zárodečných buněk (PGCs – primordial germ cells) v oplození schopné vajíčko. PGCs jsou považovány za jediný zdroj pohlavních buněk dospělého jedince, ačkoli se objevují studie naznačující existenci mitoticky aktivních zárodečných buněk ve vaječnících dospělých samic (Johnson *et al.*, 2004). Primordiální zárodečné buňky jsou velké, kulovité, rychle se dělící buňky obsahující malé množství organel ve světlé cytoplazmě (Makabe *et* Motta, 1989; Makabe *et al*., 1989). Primordiální zárodečné buňky vznikají během embryonálního vývoje ve žloutkovém váčku, odkud migrují přes zadní prvostřevo do genitálních lišt (Chiquoine, 1954). Jejich přesun ze žloutkového váčku do prvostřeva probíhá pasivně jako následek změn v uspořádání rostoucího embrya (Fujimoto *et al.*, 1977). Ze zadního prvostřeva do genitálních lišt migrují primordiální zárodečné buňky aktivně. Jejich kulovitý tvar se mění na více nepravidelný, vřetenovitý s dlouhou osou (Fukuda, 1976). Plazmatická membrána vytváří pseudopodia, v cytoplazmě se koncentrují mikrotubuly a mikrofilamenta (Fujimoto *et al.*, 1977) a buňky jsou schopny aktivního améboidního pohybu (Makabe *et al*., 1989). PGCs jsou během svého přesunu naváděny okolními somatickými buňkami (Makabe *et al*., 1989; Makabe *et al*, 1991) a komponenty extracelulární matrix (Fujimoto *et al.*, 1985). V extracelulární matrix jsou patrné změny v distribuci glykoproteinů, kolagenu, fibronektinu a lamininu (Garcia-Castro *et al.*, 1997). Integriny (Anderson *et al.*, 1999) a kadheriny (Bendel-Stenzel *et al.*, 2000) jsou významnými mediátory přesunu PGCs. Přítomnost cytokinů, fibroblastového růstového faktoru (Resnick *et al.*, 1998), tumor nekrotizujícího faktoru alfa (Kawase *et al.*, 1994) a neuregulinu beta (Toyoda-Ohno *et al.*, 1999) podporuje proliferaci PGCs. Leukemický inhibiční faktor napomáhá přežití PGCs inhibicí apoptózy (Pesce *et al.*, 1993). PGCs jsou spojené intracelulárními můstky a vykazují shodnou chromozomální konfiguraci (Baker *et* Franchi, 1967). Tyto můstky vznikají pravděpodobně v důsledku neúplného rychle probíhajícího buněčného dělení (Gondos, 1984). U prasete dochází k přesunu PGCs okolo 30. dne embryonálního vývoje (Romanovský *et al*., 1988). Přesun PGCs do místa budoucích gonád je stimulem k zahájení diferenciace vaječníků a k nástupu folikulogeneze (Wartenberg, 1989).

Primordiální zárodečné buňky diferencují v oogonie, kterým se podobají, ačkoli PGCs vykazují vyšší mitotickou aktivitu. V době, kdy je dokončena kolonizace genitálních lišt

zárodečnými buňkami, zahajují oogonie meiotické dělení. Oogonie vstupují do stádia preleptotén a následně leptotén první meiotické profáze (Franchi *et al*., 1962). Během fáze preleptotén dochází k poslední replikaci DNA a oogonie vnitřní vrstvy kůry vaječníku se transformují v oocyt (Gondos *et al.*, 1986). Oocyty mohou být v této fázi meiotického dělení, stejně jako oogonie, spojené intracelulárními můstky (Makabe *et al.,* 1992). Během stádia zygotén se párují homologní chromozómy za vzniku bivalentů složených ze čtyř chromatid. Ve stádiu pachytén dochází k výměně DNA mezi homologními chromozómy nazývané crossing-over. Ve stádiu diplotén jsou homologní chromozómy spojeny v místech překřížení, tzv. chiazmatech, která jsou výsledkem proběhlých crossing-overů. Meiotické dělení oocytů se zastaví krátce po narození ve stádiu nazývaném diktyotén, které nastává v pozdním diploténu (Wassarman, 1988).

Zárodečné buňky se v místě budoucích vaječníků mísí se somatickými buňkami, které podporují jejich následný vývoj. Tyto somatické granulózní buňky obklopují budoucí oocyty, a dávají tak vzniknout primordiálním folikulům (Vanderhyden, 2002).

Fáze růstu

Během růstové fáze oocyt ve stádiu diktyotén několikanásobně zvětší svůj objem, dochází ke změnám v uspořádání jádra, zvyšuje se počet mitochondrií, které se shromažďují okolo jádra, stejně jako Golgiho komplex. Tyto změny uspořádání organel v cytoplazmě jsou závislé pravděpodobně na činnosti mikrotubulů (Makabe *et al*., 1992).

Současně s růstem oocytu dochází k procesu folikulogeneze, tedy uzavírání oocytu do folikulu, které spočívá v přibývání a změně tvaru granulózních buněk. Vaječník samic je po narození osídlen zejména primordiálními folikuly tvořenými primárními oocyty obklopenými jednou vrstvou plochých granulózních buněk (Wassarman, 1988).

Během růstu folikulu dochází ke změně tvaru granulózních buněk z plochého na kubický. Granulózní buňky proliferují a vzniklý primární folikul je tvořen oocytem obklopeným vrstvou 11 – 20 granulózních buněk kubického tvaru (Wassarman, 1988; Hulshof *et al*., 1992). Ve vaječnících narozených prasniček se nachází přibližně 210 tisíc primárních folikulů (Prather *et* Day, 1998). Fáze růstu folikulů trvá u pohlavně dospělých samic přibližně 80 dní (Morbeck *et al*., 1992).

Sekundární folikul je tvořen oocytem obklopeným dvěma vrstvami granulózních buněk (Driancourt, 1991). V této fázi vývoje folikulu dochází k hromadění komponentů budoucí *zona pellucida* okolo oocytu. Ve stejné době se tvoří kortikální granula v cytoplazmě oocytu (Fair *et al.*, 1997b). Folikuly se stávají citlivými vůči folikuly stimulujícímu hormonu (FSH)

(Presl *et al.*, 1974). V oocytech sekundárních folikulů lze zaznamenat první známky syntézy RNA (Fair *et al.*, 1997a).

Přechod do stádia terciálního folikulu je typický pokračující proliferací a diferenciací granulózních buněk v *theca interna* a *theca externa*, tvorbou bazální membrány, diferenciací kumulárních buněk a tvorbou antrální dutiny vyplněné folikulární tekutinou (Driancourt, 1991). Na thekálních buňkách *theka interna* se objevují receptory pro luteinizační hormon (LH) (Xu *et al.*, 1995). Oocyty časných terciálních folikulů jsou transkripčně aktivní a v jejich jádře je přítomno nejméně jedno aktivní jadérko (Crozet *et al.*, 1986). Hromadění mRNA, ribozómů a polypeptidů v rostoucím oocytu je rozhodující pro pozdější embryonální vývoj oplozeného oocytu (Pavlok *et al.*, 1992).

Každý nárůst hladiny FSH je následován folikulární vlnou, tedy vytvořením skupiny rostoucích folikulů citlivých vůči FSH (Adams *et al.*, 1992). Spolu s folikulem roste i oocyt, ve kterém dochází k dalším změnám v uspořádání cytoplazmy. Mezi *zona pellucida* a cytoplazmatickou membránou oocytu se tvoří perivitelinní prostor, dochází k poklesu množství endoplazmatického retikula v ooplazmě, organely a kortikální granula putují k periferii oocytu (Fair *et al.*, 1997b).

Když rostoucí folikuly dosáhnou rozměrů 8 – 9 mm, dochází k formování skupiny dominantních folikulů, které rostou rychleji než ostatní folikuly (Savio *et al.*, 1988). Rostoucí oocyt v dominantním folikulu prodělává ultrastrukturální změny, které zahrnují nárůst množství lipidů v ooplazmě, pokles velikosti Golgiho komplexů a výraznější posun kortikálních granul k periferii. Jadérka vykazují vakuolizaci a získávají prstencovitou strukturu. Dále dochází ke zvlnění jaderné membrány, protažení buněk *corona radiata* (Assey *et al.*, 1994) a zvětšení perivitelinního prostoru (Hyttel *et al.*, 1997).

Dominantní folikuly pokračují v růstu několik dní po své selekci. Pokud nedojde ke zvýšení hladiny LH, dominantní folikuly zanikají a spouští novou vlnu rostoucích folikulů. Pokud dojde ke zvýšení hladiny LH, folikuly dále rostou a oocyty v nich obsažené podstupují proces meiotického zrání (Hyttel *et al.*, 1986). Takové oocyty dosahují u prasete velikosti 120 μm a nazýváme je meioticky kompetentními (Yanagimachi, 1988).

Fáze zrání

Oocyty získávají během fáze růstu meiotickou kompetenci, tedy schopnost obnovit proces meiózy a dokončit meiotické zrání. Meioticky kompetentní jsou pouze ty oocyty, které dosáhly 80 % velikosti plně vyvinutého oocytu. Takové oocyty se objevují v době vzniku antrální dutiny folikulu (Motlik *et al.*, 1984).

Somatické buňky folikulu mají, na rozdíl od oocytů, receptory pro gonadotropiny (Moor *et* Dai, 2001). FSH a LH hrají významnou roli v procesu získání meiotické kompetence (Fauser *et al.*, 1999), udržení prvního meiotického bloku ve fázi diktyotén (Thibault *et al.*, 1987) a při rozpadu zárodečného váčku (GVBD - germinal vesicle breakdown), při kterém dochází k rozpadu jaderné membrány za současného opuštění prvního meiotického bloku (Motlik *et* Fulka, 1976). Somatické buňky folikulu tak zprostředkovávají všechny tyto procesy probíhající v rostoucím a plně dorostlém oocytu.

Během fáze zrání dochází k přeměně plně dorostlého meioticky kompetentního oocytu v oplození schopný oocyt (Motlik *et* Fulka, 1986). Oocyt je udržován v prvním meiotickém bloku ve fázi diktyotén působením několika inhibičních faktorů, například inhibitorem zrání oocytů. Zvýšené hladiny LH v době nástupu puberty vedou k eliminaci inhibičních faktorů. Následkem toho jsou aktivovány klíčové molekuly účastnící se procesu meiotického zrání, jakými jsou cykliny, kinázy a fosfatázy (Hunter, 2000). V *in vitro* podmínkách lze navodit opuštění prvního meiotického bloku pouhým vyjmutím oocytu z folikulu, protože folikulární buňky zprostředkovávají udržení oocytu v tomto bloku (Stojkovic *et al.*, 1999).

Pro proces meiotického zrání jsou charakteristické změny v uspořádání chromatinu (Sharma *et* Chowdhury, 1998). Jádro oocytu se před zahájením meiotického zrání nachází ve fázi zárodečného váčku (GV - germinal vesicle), kdy je ohraničeno neporušenou membránou. Po zvýšení hladiny LH se jaderná membrána rozpadá, čímž je zahájeno meiotické zrání. Tento proces se nazývá rozpad zárodečného váčku (GVBD - germinal vesicle breakdown). Stádium zárodečného váčku lze u prasete rozdělit do pěti fází. Ve fázi GV0 je chromatin rozptýlen v celé jaderné oblasti. Ve fázi GV1 jsou jaderná membrána i jadérko neporušené a chromatin začíná kondenzovat za vytvoření prstencovité nebo podkovovité struktury. Fáze GV2 je podobná fázi GV1, ale dochází k tvorbě několika shluků chromatinu v blízkosti jaderné membrány. Ve fázi GV3 dochází k rozptýlení chromatinových shluků a vláken po celé nukleoplazmě. Ve fázi GV4 jsou stále přítomny shluky a vlákna chromatinu, ale jaderná membrána již není zřetelná a jadérko zcela mizí (Motlik *et* Fulka, 1976; Guthrie *e*t Garrett, 2000).

V závěru procesu rozpadu zárodečného váčku, ve stádiu diakineze, dochází ke shlukování kondenzovaného chromatinu. Vytváří se dělící vřeténko, na jehož mikrotubuly se pomocí kinetochorů přichycují páry chromozómů. V metafázi I se chromozómy řadí v ekvatoriální rovině. Centromery chromozómů jsou orientovány k opačným pólům vřeténka. V anafázi I dochází k rozchodu homologních chromozómů k protilehlým pólům buňky. V telofázi I dojde k vydělení prvního pólového tělíska, které obsahuje jednu sadu chromozómů. Meióza I

přechází plynule v meiózu II bez replikace DNA. Do meiózy II tak vstupují buňky s poloviční sadou chromozómů. Meióza se zastavuje v druhém bloku ve fázi metafáze II, čímž končí proces meiotického zrání (Wassarman, 1988). Oocyt v metafázi II může vystoupit z druhého meiotického bloku a dokončit proces meiózy pouze po aktivaci oplozením spermií nebo partenogenezí.

Regulace meiotického zrání

Úspěšný průběh meiotického zrání a vznik oplození schopného vajíčka vyžaduje složitý systém regulace, který je zajištěn změnami v aktivitě řady proteinů.

Opětovné zahájení meiózy je závislé na snížení hladiny cyklického adenosin monofosfátu (cAMP). Pokud jsou hladiny cAMP v oocytu vysoké, dochází k aktivaci protein kinázy A. Aktivní protein kináza A blokuje aktivitu fosfatázy cdc25, která je nezbytná pro aktivaci faktorů klíčových pro zahájení meiotického zrání (Liang *et al.*, 2007). Cyklický AMP je syntetizován v oocytu a kumulárních buňkách (Cho *et al*., 1974). Pokles koncentrace cAMP je zapříčiněn potlačením aktivity enzymu adenylát cyklázy a aktivací fosfodiesteráz štěpících dvojnou vazbu cAMP (Dekel *et* Beers, 1980). Dalším důležitým faktorem způsobujícím snížení hladin cAMP v oocytu je přerušení spojů typu *gap juction* mezi oocytem a kumulárními buňkami obklopujícími oocyt během procesu kumulární expanze. Těmito spoji proudí molekuly cAMP do oocytu a inhibují tak meiotické zrání. Hladina cAMP zůstává nízká po celou dobu meiotického zrání (Liang *et al*., 2007).

M-fázi podporující faktor (MPF – M-phase promoting factor) je proteinový komplex protein kinázy a cyklinu zodpovědný za rozpad zárodečného váčku a zahájení meiotického zrání oocytu (Masui *et* Markert, 1971; Sorensen *et al.*, 1985). Regulační podjednotkou MPF je cyklin B, katalytickou podjednotkou je cyklin-dependentní protein kináza 1 (Cdk 1).

Cdk 1 je serin/threonin protein kináza ze skupiny tyrosinových cyklin-dependentních kináz. Fosforylací příslušných proteinů navozuje kondenzaci chromozómů, rozpad jaderného obalu a reorganizaci mikrotubulů při tvorbě dělícího vřeténka (Alberts *et al.,* 1998). Aktivní Cdk 1 musí být ve vazbě s cyklinem B a musí být fosforylována a defosforylována na specifických místech. Cdk 1 je aktivována náhle na konci interfáze odstraněním inhibičního fosfátu z její molekuly. Aktivní MPF komplex aktivuje na základě pozitivní zpětné vazby další MPF komplexy, čímž dochází k rychlému nárůstu kinázové aktivity (Nebreda *et al.,* 1995) .

Po dosažení metafáze I dochází k přechodnému poklesu aktivity MPF. Cyklin B je degradován ubikvitin–dependentním proteolytickým systémem. Na základě kovalentní vazby ubikvitinu s cyklinem B dochází k degradaci cyklinu B v proteazómech. Aktivace Cdk 1

iniciuje s určitým zpožděním ubikvitinaci cyklinu B, čímž v konečném důsledku inaktivuje sebe sama (Glotzer *et al.*, 1991).

Reaktivace MPF vede k dosažení metafáze II a dokončení meiotického zrání (Hampl *et* Eppig, 1995). Aktivita MPF zůstává vysoká až do aktivace oocytu spermií a podílí se na udržení druhého meiotického bloku (Yanagimachi, 1988).

Mitogenem aktivovaná protein kináza (MAPK – mitogen-activated protein kinase) patří do skupiny serin/threonin protein kináz. MAPK je aktivní, je-li fosforylována na aminokyselinových zbytcích threoninu a tyrosinu (Alberts *et al*., 1998). Signální dráha proteinů Mos a MEK vede k aktivaci MAPK. Do regulace této signální kaskády je zapojen FSH (Li *et al*., 2002), cAMP (Liang *et al*., 2005) a MPF (Fan *et al*., 2002). Aktivita MAPK v prasečích oocytech narůstá krátce před GVBD a zůstává vysoká po celou dobu meiotického zrání (Lee *et al*., 2000).

Aktivita MAPK není pro GVBD prasečích oocytů nezbytná, nicméně na tomto procesu participuje prostřednictvím aktivace MPF (Ohashi *et al*., 2003). MAPK je nezbytná pro kondenzaci a segregaci chromozómů (Kishimoto, 2003), podílí se na fosforylaci mikrotubuly organizujícího centra a formaci dělícího vřeténka (Fan *et al*., 2002; Li *et al*., 2002). MAPK fosforyluje jaderné laminy a zabraňuje tak udržení intaktní jaderné membrány (Inoue *et al*., 1998). Její aktivita brání průběhu interfáze mezi meiózou I a II a s ní spojené replikaci chromozómů (Kishimoto, 2003; Fan *et* Sun, 2004). Ribozomální s6 kináza $p90^{rsk}$ je substrátem MAPK, díky kterému se MAPK účastní opětovné syntézy MPF při přechodu oocytu z meiózy I do meiózy II (Gross *et al*., 2000). MAPK zajišťuje asymetrickou cytokinezi a vydělení prvního pólového tělíska na konci meiotického zrání (Tong *et al*., 2003).

Cytostatický faktor (CSF) je proteinový komplex zodpovědný za udržení oocytu v metafázi II po ukončení procesu meiotického zrání (Masui *et* Markert, 1971). Aktivita CSF vzrůstá již po dokončení meiózy I a zůstává vysoká do konce meiotického zrání (Takakura *et al*., 2005). CSF brání polyubikvitinaci cyklinu B, a tím jeho proteolytické degradaci (Maller *et al*., 2001). Komplex MPF je tak stabilní a jeho aktivita konstantní (Maller *et al*., 2002). Aktivní MPF udržuje na principu pozitivní zpětné vazby vysokou aktivitu CSF (Kishimoto, 2003). Aktivita CSF je potlačena až po aktivačním stimulu - oplozením oocytu spermií nebo partenogenetickou aktivací (Fan *et al*., 2002).

Proces meiotického zrání oocytu může být pozitivně ovlivněn gasotransmitery. Gasotransmitery jsou buněční poslové, mezi které patří oxid dusnatý a sulfan (Wang, 2002). V případě oxidu dusnatého již byla úloha v procesu meiotického zrání oocytu prokázána (Chmelikova *et al*., 2010).

Kumulární expanze

Kumulární expanze probíhá současně s meiotickým zráním oocytu. Gonadotropní hormony stimulují proces kumulární expanze (Dekel *et al*., 1979). Gonadotropiny ovlivňují metabolismus granulózních buněk, které se účastní parakrinní regulace procesu kumulární expanze přímo ve folikulu (Motlík *et al*., 1998). Folikulární tekutina funguje jako mediátor v komunikaci mezi granulózními buňkami a kumulo-oocytárními komplexy (COCs - cumulus-oocyte complexes) a vykazuje tak schopnost indukce kumulární expanze (Nakayama *et al*., 1996). Kumulární buňky disponují receptory pro gonadotropiny a zprostředkovávají tak jejich účinky oocytu (Downs *et al*., 1986).

Kumulární buňky se podílejí na zisku meiotické a vývojové kompetence oocytu (Sirard *et al*., 1988; Qian *et al*., 2003), jejich přítomnost je nezbytná pro vydělení prvního pólocytu a ovlivňuje přežitelnost zrajících oocytů (Ju *et* Rui, 2012). Kumulární buňky se dále podílí na ovulaci zralého oocytu a jeho oplození spermií (Chen *et al*., 1993). Kumulární buňky brání předčasnému tvrdnutí *zona pellucida* (Downs *et al*., 1986) a regulují energetický metabolismus oocytu syntézou mastných kyselin a lipolýzou (Auclair *et al*., 2013).

Kumulární buňky bezprostředně obklopující oocyt syntetizují komponenty extracelulární matrix, což vede ke zvětšování kumulárního obalu oocytu a k oddalování kumulárních buněk. Agregáty glykosaminoglykanů (GAGs) a membránových proteinů kumulárních buněk tvoří proteoglykanové komplexy a představují stěžejní komponentu extracelulární matrix expandovaného kumulu (Moscatelli *et* Rubin, 1974). Hyaluronová kyselina (HA) jako významný GAG expandovaného kumulu přispívá k oddalování kumulárních buněk během kumulární expanze (Nakayama *et al*., 1996).

Pro regulaci meiotického zrání kumulárními buňkami jsou klíčové spoje typu *gap junction*. Spoje *gap junction* umožňují prostup pouze molekulám o velikosti do 1 kDa (Moor *et al*., 1980). Těmi proudí z kumulárních buněk do oocytu malé signální molekuly jako cAMP, cyklický guanosin monofosfát (cGMP) a Ca^{2+} (Yanagimachi, 1988). Tyto molekuly regulují meiotické zrání oocytů tím, že ovlivňují aktivitu protein kináz jako MPF a MAPK (Tatemoto *et* Terada, 1998). Kumulární expanze je doprovázená endocytózou proteinů buněčných spojů, které brání toku signálních molekul z kumulárních buněk do oocytu (Chen *et al*., 1990). Na membráně kumulárních buněk a v oocytu jsou receptory pro hyaluronovou kyselinu (Kimura *et al*., 2002). Tyto receptory jsou intenzivně syntetizovány po hormonální stimulaci gonadotropiny, během meiotického zrání (Yokoo *et al*., 2002). Receptory jsou po navázání HA aktivovány a fosforylují proteiny spojení typu *gap junction*, uzavírají tak tento spoj a

brání přítoku signálních molekul jako je cAMP do oocytu (Yokoo *et al*., 2010). Produkce HA během procesu kumulární expanze pozitivně koreluje s úspěšností meiotického zrání *in vitro* (Qian *et al*., 2003).

Oocyty se podílejí na regulaci kumulární expanze uvolňováním faktoru podporujícího kumulární expanzi během meiotického zrání (Eppig *et al*., 1993). Syntéza HA kumulárními buňkami je podmíněna přítomností oocytu a závisí na jeho kvalitě. Pouze plně dorostlý, meioticky kompetentní oocyt je schopný dostatečně stimulovat kumulární expanzi (Tirone *et al*., 1993). Odstranění oocytu brání produkci HA kumulárními buňkami (Buccione *et al.*, 1990). Společná kultivace kumulárních obalů s oocyty zbavenými kumulárních buněk tento efekt zvrátí, z čehož vyplývá, že schopnost oocytu ovlivňovat produkci HA není závislá na spojích *gap junction* (Vanderhyden *et al*., 1990). Oocyt reguluje funkci kumulárních buněk, které zpětně ovlivňují jeho meiotické zrání a nabytí vývojové kompetence.

Aktivace zralého oocytu a časný embryonální vývoj

Vývojová kompetence oocytu je definována jako jeho schopnost být oplodněn a vyvíjet se ve zdravé embryo vedoucí ke vzniku nového, životaschopného jedince. Oocyt nabývá vývojové kompetence během meiotického zrání. Nabytí vývojové kompetence je závislé na transkripční inaktivitě zrajícího oocytu a na kondenzaci chromatinu ve zrajícím oocytu (Christians *et al.*, 1999). Transkripčně inaktivní oocyty vykazují vyšší expresi proteinu Oct4, která pozitivně koreluje s úspěšností embryonálního vývoje (Zuccotti *et al.*, 2008). Protein DAZL je dalším maternálním faktorem nezbytným pro nabytí vývojové kompetence oocytu. Oocyty vykazující sníženou expresi tohoto faktoru blokují svůj vývoj po oplození ve stádiu dvoubuněčného embrya (Chen *et al*., 2011).

Schopnost mobilizovat zásoby Ca^{2+} představuje klíčový faktor ovlivňující schopnost oocytu být aktivován (Vincent *et al*., 1992). Spermie penetrující oocyt způsobí nárůst hladin Ca^{2+} vnesením fosfolipázy C zeta (PLCζ - phospholipase C zeta). PLCζ katalyzuje hydrolýzu fosfatidylinositol-4,5-bisfosfátu na inositol trifosfát a diacylglycerol (Cox *et al*., 2002). Inositol trifosfát se váže na své receptory na endoplazmatickém retikulu a spouští tak oscilace v hladinách Ca^{2+} (Markoulaki *et al*., 2003). Oscilace v hladinách Ca^{2+} vedou k aktivaci kalmodulin-dependentní kinázy II a následně ke snížení aktivity CSF (Fan *et al.*, 2002). Nárůst intracelulárních hladin Ca^{2+} zároveň zvyšuje aktivitu anafázi podporujícího komplexu/cyklozómu, který je zodpovědný za degradaci cyklinu B, a tím inaktivaci MPF (Yamamoto *et al.*, 2005). Jako prevence polyspermie dochází k ztvrdnutí *zony pellucidy* a

zvětšení perivitelinního prostoru (DeMeestere *et al.*, 1997). Meióza II je dokončena vydělením druhého pólocytu (Lopata *et al*., 1980).

Průběh prvního buněčného cyklu oplozeného oocytu závisí na maternální mRNA nahromaděné během profáze prvního meiotického dělení, kdy oocyt prochází fází růstu (Bachvartova *et* De Leon, 1980). Během prvního embryonálního buněčného cyklu se replikuje maternální a paternální DNA odděleně (Bomar *et al*., 2002). Maternální a paternální prvojádro prochází rozpadem jaderné membrány. Splývají během formace metafázní figury a dělícího vřetene (Donahue, 1972).

2.2 Proces stárnutí oocytů

V případě, že jsou oocyty po dokončení fáze zrání vystaveny prodloužené kultivaci, nastává proces označovaný jako stárnutí oocytu. Pro proces stárnutí jsou charakteristické mnohé morfologické změny. Mění se struktura cytoplazmatické membrány, která tvoří klkovité výběžky a může tak přecházet až do perivitelinního prostoru (Szollosi, 1971). *Zona pellucida* tvrdne a získává dlaždicovitou strukturu tvořenou shluky granulo-fibrilárního materiálu (Longo, 1981). V mitochondriích stárnoucích oocytů dochází ke změnám membránového potenciálu a bobtnání mitochondriální matrix. Během stárnutí oocytu dochází ke zvětšování perivitelinního prostoru a posunu a degeneraci prvního pólového tělíska (Miao *et al.*, 2004). Kortikální granula stárnoucího oocytu se přemísťují a podléhají částečné exocytóze i spontánně, aniž by došlo k fertilizaci (Szollosi, 1971). Dělící vřeténko se zkracuje, může být multipolární a dochází tak k poruše procesu segregace chromozómů (George *et al.*, 1996). Během stárnutí oocytu také dochází k zániku centrozomálních struktur a tak ke ztrátě integrity mikrotubulů (Sun *et* Schatten, 2007). Exprese proteinů udržujících stabilitu dělícího vřeténka a motorového proteinu kinezinu EG5 je rovněž narušena (Hall *et al.*, 2007). Cytoplazmatické mikrotubuly podléhají depolymerizaci (Longo, 1974). Ve stárnoucích oocytech dochází ve zvýšené míře k předčasné separaci chromozómů a tak ke zvýšenému výskytu aneuploidií (Mailhes *et al.*, 1998). Dále dochází k fragmentaci a ztrátě chromozómů a ke shlukování a separaci chromatid (Rodman, 1971).

Během stárnutí oocytů dochází ke změnám v aktivitě MPF a MAPK. Po dokončení meiotického zrání je nezbytné zachování vysoké aktivity MPF a MAPK pro udržení oocytu v druhém meiotickém bloku (Kikuchi *et al.*, 2000). Postupný pokles aktivity MPF a MAPK během prodloužené kultivace oocytů vede k výskytu nežádoucích jevů, jako jsou spontánní partenogenetická aktivace a fragmentace oocytů (Kikuchi *et al.*, 2000). Bylo prokázáno, že udržení vysoké aktivity MPF prostřednictvím přidání kofeinu do kultivačního média potlačuje

spontánní partenogenetickou aktivaci a fragmentaci oocytů (Kikuchi *et al.,* 2002). Dojde-li k oplození oocytu, MPF je deaktivován degradací cyklinu B a defosforylací Cdk 1 na threoninu-161. Naproti tomu během prodloužené kultivace oocytů zůstávají obě podjednotky MPF spojené a Cdk 1 je fosforylována na threoninu-14 a tyrosinu-15, čímž je MPF deaktivován (Kikuchi *et al.,* 2002).

Během prodloužené kultivace oocytů dochází k nárůstu hladin ROS, zejména peroxidu vodíku, peroxynitritu a superoxidového aniontu (Takahashi *et al.*, 2003). Současně dochází k vyčerpávání zásob intracelulárních antioxidantů, například glutationu (Boerjan *et* de Boer, 1990), a stárnoucí oocyty jsou tak vystaveny oxidativnímu stresu. ROS působí jako inhibitory fosfatázy cdc25 zodpovědné za aktivační defosforylaci MPF (Brisson *et al.*, 2007) a zároveň stimulují aktivitu kináz Wee1 a Myt1, které udržují MPF v neaktivní formě (Kikuchi *et al.*, 2000). Zvýšené hladiny ROS ve stárnoucích oocytech ovlivňují homeostázu Ca^{2+} tím, že ovlivňují vápníkové kanály, kalmodulin (Gao *et al.*, 2005) a Ca^{2+}-dependentní ATPázy endoplazmatického retikula (Rohn *et al.*, 1993). Pokud dojde k oplození stárnoucího oocytu, objevují se abnormální oscilace Ca^{2+}, které přispívají k nástupu apoptózy (Takahashi *et al.*, 2000). Ionty Ca^{2+} se uvolňují s výrazně nižší amplitudou a s podstatně vyšší frekvencí než v oocytech, které nebyly vystavené prodloužené kultivaci (Igarashi *et al*., 1997).

Během stárnutí oocytů dochází v mitochondriích k narušení mitochondriální DNA (Sohal *et* Dubey, 1994), změně membránového potenciálu (Liu *et al.*, 2000) a poklesu produkce ATP (Chi *et al.*, 1988). Poškození mitochondrií může vést až k uvolnění cytochromu C z vnitřní mitochondriální membrány. Uvolněný cytochrom C aktivuje kaspázy (Takai *et al.*, 2007), proteiny z rodiny cystein proteáz, které štěpí široké spektrum buněčných substrátů a stojí tak na počátku apoptotické signální dráhy (Lamkanfi *et al.*, 2002). Během stárnutí oocytů dochází rovněž k nárůstu exprese proapoptotických faktorů (Chaube et al., 2007) a poklesu exprese antiapoptotických faktorů v oocytech (Takahashi *et al.*, 2009), což rovněž přispívá k nástupu apoptózy.

Stárnoucí oocyty tak vykazují sníženou oplozovací schopnost (Lanman, 1968) a vnímavost vůči aktivačním stimulům (Szollosi, 1971), zvýšený výskyt polyspermního oplození (Badenas *et al.*, 1989), chromozomálních aberací, částečnou exocytózu kortikálních granul (Szollosi, 1971), epigenetické změny (Liang *et al.*, 2008) a zhoršený embryonální vývoj (Lanman, 1968).

2.3 Sulfan jako buněčný posel

Gasotransmitery jsou malé plynné molekuly, které plní v organismu signální funkci. Patří mezi ně oxid dusnatý, oxid uhelnatý a sulfan. Tyto plynné molekuly byly dříve považovány jen za látky znečišťující ovzduší a vykazující toxické účinky v organismu. Později bylo zjištěno, že v nízkých koncentracích plní úlohu signálních molekul v řadě procesů v organismu, jako jsou vasodilatace, vedení nervového vzruchu a imunitní odpověď. Gasotransmitery volně přecházejí přes cytoplazmatickou membránu cílových buněk a jejich účinek je tak nezávislý na přítomnosti membránového receptoru. Jsou produkovány endogenně aktivitou enzymů a jejich produkce je organismem regulována (Wang, 2002).

Sulfan je bezbarvý plyn silného zápachu. V organismu je sulfan endogenně produkován aktivitou pyridoxal-5‘-fosfát dependentních enzymů cystathionin-beta-syntázy (CBS – cystathionine-beta-synthase) a cystathionin-gama-lyázy (CSE – cystathionine-gamma-lyase) (Wang, 2002) a pyridoxal-5‘-fosfát independentního enzymu 3-merkaptopyruvát-sulfurtransferázy (3-MPST – 3-mercaptopyruvate-sulfurtransferase) (Shibuya *et al.*, 2009a), které využívají jako substrát aminokyselinu L-cystein. Většina fyziologicky produkovaného sulfanu vzniká aktivitou CBS a CSE. V některých tkáních je k produkci sulfanu zapotřebí přítomnosti obou enzymů, v jiných stačí přítomnost pouze jednoho z nich (Wang, 2002).

Fyziologicky se koncentrace endogenního sulfanu pohybuje od 50 μM do 160 μM (Abe *et* Kimura, 1996). Sulfan vykazuje negativní zpětnou vazbu na aktivitu enzymů zodpovědných za jeho produkci (Wang, 2002). K udržení fyziologických koncentrací sulfanu v organismu je zapotřebí mechanismů, které sulfan odbourávají. Sulfan je oxidován v mitochondriích na thiosíran, který je dále přeměněn na síran. Druhou cestou odstranění sulfanu z organismu je jeho metylace pomocí thiol-S-metyltransferázy na methanethiol a dimetylsulfid (Furne *et al.*, 2001). Sulfan je produkován a plní fyziologickou funkci například v centrální nervové soustavě (Abe *et* Kimura, 1996), cévní soustavě (Zhao *et al.*, 2001), samčí reprodukční soustavě (Srilatha *et al*., 2007), ale také v samičí reprodukční soustavě (Srilatha *et al*., 2009).

Fyziologické účinky sulfanu byly jako první popsány v nervovém systému (Goodwin *et al.*, 1989; Warenycia *et al.*, 1989). Vysoké koncentrace endogenního sulfanu byly zjištěny v mozku potkanů, skotu i člověka (Eto *et al.*, 2002). Dlouhodobá expozice sulfanu zvyšuje uvolňování neurotransmiterů v mozku potkaních mláďat (Skrajny *et al.*, 1992; Roth *et al.*, 1995). Donor sulfanu potlačuje uvolňování kortikotropin-uvolňujícího hormonu v hypotalamu potkanů (Dello Russo *et al.*, 2000). Cílem sulfanu v nervovém systému jsou iontové kanály a NMDA receptory. Sulfan ovlivňuje v neuronech aktivitu napěťově a tetradotoxinem řízených iontových kanálů pro Na^+ (Warenycia *et al.*, 1989). Váže se také na ATP-řízené iontové

kanály pro K^+ a způsobuje tak hyperpolarizaci cytoplazmatické membrány neuronů (Reiffenstein *et al.*, 1992). Svou vazbou na NMDA receptory se sulfan účastní procesu dlouhodobé potenciace v hipokampu a podílí se tak na procesech učení a utváření paměti (Abe *et* Kimura, 1996). Interakce sulfanu a NMDA receptorů je pravděpodobně zprostředkovaná aktivací signální dráhy protein kinázy A. Donor sulfanu zvyšuje produkci cAMP v neuronech mozku a mozečku a gliových buňkách potkana (Kimura *et al.*, 2000).

Sulfan působí jako signální molekula také v kardiovaskulárním systému. Exprese CSE a endogenní produkce sulfanu byla prokázána v portální žíle a aortě potkana (Hosoki *et al.*, 1997). Později byla přítomnost CSE u potkana prokázána také v dalších cévách s nejvyšší expresí v plicní tepně (Zhao *et al.*, 2001). Sulfan snižuje krevní tlak a způsobuje vasorelaxaci aorty a portální žíly u potkana (Hosoki *et al.*, 1997; Zhao *et al.*, 2001). Donor sulfanu snižuje krevní tlak u hypertenzních myší (Al-Magableh *et al.*, 2015). Na rozdíl od oxidu dusnatého a oxidu uhelnatého sulfan nepůsobí na hladkosvalové buňky cév přes signální dráhu cGMP (Wang, 2002). Cílem sulfanu v cévní soustavě jsou ATP-řízené iontové kanály pro K^+ (Zhao *et al.*, 2001).

Sulfan zasahuje jako signální molekula také do regulace reprodukčních funkcí. Exprese enzymů produkujících sulfan byla prokázána v samčí i samičí reprodukční soustavě. U samců sulfan ovlivňuje erekci penisu tím, že přispívá k relaxaci hladkého svalstva cév a má pozitivní vliv na tlak v topořivém tělísku (Srilatha *et al.*, 2006; Jupiter *et al.*, 2015). Enzymy CBS a CSE a jejich mRNA byly detekovány také v lidské erektilní tkáni (D'Emmanuele *et al.*, 2009). Ve varlatech potkana byl enzym CBS detekován v Leydigových, Sertoliho a zárodečných buňkách, přítomnost CSE byla prokázána jen v Sertoliho a nezralých zárodečných buňkách (Sugiura *et al.*, 2005).

Ve folikulárních buňkách myších vaječníků byla prokázána vysoká exprese CBS (Liang *et al.*, 2006). Myši se zablokovaným genem pro CBS měly nižší počet vyvíjejících se folikulů a nepravidelný estrální cyklus (Guzman *et al.*, 2006). Potlačení exprese enzymu CBS v granulózních buňkách inhibuje meiotické zrání oocytu (Liang *et al.*, 2007). CBS a CSE jsou exprimovány v lidském vejcovodu, uvolněný sulfan spouští kontrakce vejcovodu, které zajišťují posun embrya (Ning *et al.*, 2014). Enzymy CBS a CSE byly detekovány v děloze a placentě potkana (Patel *et al.*, 2009). Endogenní produkce sulfanu prostřednictvím enzymu CSE je nezbytná pro správné prokrvení placenty a působí tak proti rozvoji preeklampsie u žen (Wang *et al.*, 2013). Exprese CBS a CSE je potlačena v placentě žen postižených preeklampsií (Hu *et al.*, 2015). Sulfan tlumí kontrakce děložní svaloviny u potkana (Sidhu *et*

al., 2001) a člověka (Hu *et al.*, 2011). Endogenní produkce sulfanu prostřednictvím enzymu CSE v pochvě potkana zvyšuje lubrikaci poševní sliznice (Sun *et al.*, 2016).

Sulfan vykazuje v řadě typů tkání antioxidační a antiapoptotické účinky. Sulfan působí na cílové proteiny jejich sulfhydratací, kdy dochází k výměně skupiny –SH za skupinu –SSH v molekule bílkoviny (Mustafa *et al.*, 2009). Proces sulfhydratace představuje jeden z mechanismů antioxidačního účinku sulfanu v organismu (Yang *et al.*, 2013; Xie *et al.*, 2014). Sulfan ochraňuje buňky před ischemicko-reperfúzním poškozením prostřednictvím své schopnosti snižovat oxidativní stres, inhibuje apoptózu, modifikuje mitochondriální funkce a aktivuje antioxidativní enzymy (Moody *et* Calvert., 2011). Sulfan působí v nervových buňkách antioxidačně a antiapoptoticky a je tak vhodný k léčbě neurodegenerativních onemocnění (Xue *et al.*, 2015). Sulfan prostřednictvím svých antioxidačních účinků chrání neurony před progresí Alzheimerovy choroby (Whiteman *et al.*, 2004). Sulfan ochraňuje nervové buňky před mrtvicí u potkanů (Wei *et al.*, 2015). Sulfan ochraňuje jaterní buňky před iatrogenně indukovaným oxidativním stresem a zánětlivými změnami (Morsy *et al.*, 2010). Zvýšená aktivita CSE a produkce sulfanu ochraňuje kardiomyocyty před oxidativním stresem a apoptózou (Gong *et al.*, 2015). Donor sulfanu zlepšuje funkci kardiomyocytů postižených ischémií (Sun *et al.*, 2015). Sulfan ochraňuje prostřednictvím svých antioxidačních účinků srdeční buňky myší před poškozením cigaretovým kouřem (Zhou *et al.*, 2015). Sulfan působí antiapoptoticky na srdeční buňky a buňky nadledvin prostřednictvím snížení aktivity MAPK (Guo *et al.*, 2013; Li *et al.*, 2016). Sulfan potlačuje také aktivitu c-Jun-N-terminální kinázy (JNK) a působí tak antiapoptoticky v jaterních buňkách potkana (Yuan *et al.*, 2016).

Přestože je sulfan v organismu endogenně produkován aktivitou sulfan uvolňujících enzymů, také exogenně dodaný donor sulfanu může mít pozitivní účinky na organismus. Jako exogenní zdroj sulfanu mohou v organismu působit například česnekové deriváty (Louis *et al.*, 2012).

2.4 Účinky česnekových derivátů v organismu

Biosyntéza a metabolismus česnekových derivátů

Česnek je všeobecně známý svými pozitivními účinky v organismu. Česnek stimuluje imunitní systém, má antimikrobiální, antivirové, antiparazitární (Iciek *et al.*, 2009), hepatoprotektivní (Banerjee *et al*., 2003) a neuroprotektivní účinky (Borrelli *et al.*, 2007) a snižuje oxidativní stres v buňkách. Česnek potlačuje proliferaci některých typů nádorových buněk (Banerjee *et al.*, 2003), snižuje hladiny triacylglycerolů a cholesterolu v krevní plazmě,

snižuje krevní tlak a brání rozvoji aterosklerózy (Iciek *et al.*, 2009). Česnek rovněž snižuje hladiny glukózy v plazmě, čímž působí proti rozvoji diabetu mellitu (Banerjee *et al.*, 2003).

Za většinu pozitivních účinků česneku v organismu jsou zodpovědné sirné sloučeniny. Sirná sloučenina gama-glutamyl cystein, obsažená v neporušených česnekových palicích, podstupuje dvě významné reakce. Hydrolýzou a oxidací je přeměňován na alliin (S-allyl cystein sulfoxid). Dlouhodobou extrakcí je účinkem enzymu gama-glutamyl transpeptidázy přeměněn na S-allyl cystein (SAC) (Corzo-Martinez *et al.*, 2007).

Alliin je sirná aminokyselina a netěkavá sloučenina bez zápachu a je hlavní složkou neporušené česnekové palice (Stoll *et* Seebeck, 1948). Rozdrcením česnekových palic vzniká čerstvý česnekový extrakt, ve kterém je alliin přeměn na kyselinu sulfonovou, pyruvát a amoniak (Amagase, 2006). Tato reakce je podmíněna přítomností enzymu alliinázy obsaženého ve vakuolách česnekových palic. Allináza je klíčový enzym, který katalyzuje přeměnu cysteinových sulfoxidů na thiosulfináty (Cavallito *et* Bailey, 1944). Allináza potřebuje ke své aktivitě S-metyl-L-cystein jako substrát (Mazelis *et* Crews, 1968). Pyridoxalfosfát stimuluje aktivitu allinázy jako její kofaktor (Amagase, 2006). Optimální pH pro činnost allinázy je 6,5. Klesne-li pH pod 3,6, což je situace typická pro obsah žaludku, je allináza úplně a irreverzibilně inhibována (Lawson *et* Hughes, 1992). Po perorálním podání alliinu myším byla jeho přítomnost prokázána v žaludku, střevě a játrech, aniž by byl alliin dále metabolizován na další sirné sloučeniny (Lachmann *et al.*, 1994; Egenschwind *et al.*, 1992). Enzym alliináza přestává účinkovat při 60 ˚C, proto tepelně opracovaný česnek obsahuje především alliin.

Kyselina sulfonová vzniklá z alliinu účinkem enzymu allinázy je velmi reaktivní a podstupuje kondenzační reakci s jinou molekulou téže kyseliny za vzniku allicinu (diallyl thiosulfinátu) (Lanzotti, 2006). Allicin se v organismu váže na proteiny a mastné kyseliny v plazmatických membránách, a je tak vychytán dříve, než by mohl být vstřebán do krve (Freeman *et* Kodera, 1995). Allicin představuje meziprodukt v tvorbě dalších sirných sloučenin, které disponují biologickou aktivitou.

Allicin se v roztoku rychle rozkládá na v tucích rozpustný diallyl sulfid (DAS) a polysulfidy, zejména diallyl disulfid (DADS) a diallyl trisulfid (DATS). Polysulfidy jsou obsažené v drceném česneku a v česnekovém oleji (Miething, 1988). Česnekový olej obsahuje přibližně 20 různých sulfidů, některé z nich jsou zodpovědné za typickou chuť a vůni česneku (Lawson *et al.*, 1991). DATS je nejvíc zastoupený sulfid v čerstvém česnekovém oleji (Miething, 1988). Při pokojové teplotě a době rozkladu 20 hodin je vzájemný poměr v tucích

rozpustných sloučenin vzniklých z allicinu následující: 66,67 % DADS, 14,6% DATS a 13,3 % DAS (Brodnitz *et al.*, 1971).

Vedle čerstvého česnekového extraktu vzniklého drcením česnekových palic lze česnek zpracovat metodou dlouhodobé extrakce v 15-20% etanolu, která vede ke vzniku vyzrálého česnekového extraktu (AGE – aged garlic extract). AGE obsahuje výrazně nižší množství allicinu ve srovnání s čerstvým česnekem. V průběhu dlouhodobé extrakce vznikají další sirné sloučeniny. Gama-glutamylcystein, přítomný v neporušených česnekových palicích, je během procesu dlouhodobé extrakce přeměněn na sirnou aminokyselinu S-allyl cystein (SAC) (Amagase, 2006; Colin-Gonzalez *et al.*, 2012). SAC je bezbarvá krystalická látka bez zápachu a je stabilní v pevném skupenství i ve vodném roztoku při neutrálním či mírně kyselém pH (Kodera *et al.*, 2002). Po konzumaci AGE byla prokázána přítomnost SAC v krvi v koncentraci závislé na dávce (Rosen *et al.*, 2001; Steiner *et* Li, 2001).

Antioxidační a antiproliferativní účinky česnekových derivátů v organismu

Gama-glutamyl cystein obsažený v česnekových palicích je hydrolýzou a oxidací přeměněn na alliin, který se při porušení česnekových palic přeměňuje na nestabilní allicin, který se dále rozkládá na v tucích rozpustný DAS a polysulfidy. Alliin a z něj odvozené česnekové deriváty vykazují v organismu dvojí aktivitu; působí antiproliferativně a antioxidačně v závislosti na aplikované dávce a typu tkáně.

Dlouhodobé podávání alliinu snižuje peroxidaci lipidů a zvyšuje aktivitu antioxidačních enzymů (Banerjee *et al.*, 2003). Alliin je hlavní složkou tepelně opracovaného česneku a sušeného česnekového prášku. Tepelně opracovaný česnek disponuje schopností vychytávat volné radikály (Kourounakis *et* Rekka, 1991) a ochraňovat molekuly lipidů a LDL před oxidací (Sheela *et* Augusti, 1995). Sušený česnekový prášek vykazuje stejné schopnosti a navíc ochraňuje buňky před ischemicko-reperfúzním poškozením (Rietz *et al.*, 1995) a chemickou cytotoxicitou (Ciplea *et* Richter, 1998).

Allicin je hlavní složkou čerstvého česnekového extraktu, který vykazuje schopnost vychytávat volné radikály s účinkem závislým na dávce (Prasad *et al.*, 1996), zvyšovat aktivitu endogenních antioxidantů (Banerjee *et al.*, 2001), potlačovat peroxidaci lipidů a oxidaci LDL (Lau, 2001). Čerstvý česnekový extrakt ochraňuje buňky před ischemicko-reperfúzním poškozením (Batirel *et al.*, 1996) a cytotoxickými sloučeninami (Kagawa *et al.*, 1986), inhibuje cytochrom P450 (Wang *et al.*, 1996) a zvyšuje produkci oxidu dusnatého, který v buňkách působí jako antioxidant (Das *et al.*, 1995). Čerstvý česnekový extrakt chrání mozkové a srdeční buňky před oxidativním stresem indukovanou ischémií (Batirel *et al.*,

1996; Banerjee *et al.*, 2003) a snižuje peroxidaci lipidů v srdci, játrech a ledvinách (Banerjee *et al.*, 2001; Banerjee *et al.*, 2002). Allicin vykazuje také antioxidativní efekt v *in vitro* podmínkách. Allicin reaguje s cysteinem a glutationem (Rabinkov *et al.*, 2000). Produkty těchto reakcí působí v organismu jako antioxidanty. Allicin a jeho deriváty mohou ovlivňovat aktivitu proteinů obsahujících thiolovou skupinu jejich S-thiolací. S-thiolace ovlivňuje aktivitu řady proteinů účastnících se procesů intracelulární signalizace a je považována za jeden z antioxidačních mechanismů buňky (Pinto *et al.*, 2006).
Polysulfidy jsou hlavní složkou česnekového oleje. Česnekový olej ochraňuje kardiomyocyty před oxidativním stresem způsobeným nadměrným množstvím cholesterolu v krvi. Ochraňuje tak kardiomyocyty před apoptózou navozenou zvýšenou konzumací tučné stravy (Cheng, 2013). Česnekový olej, DADS a DATS potlačují expresi kaspázy-3 a inhibují tak apoptotickou signální dráhu v kardiomyocytech diabetických potkanů (Huang *et al.*, 2013). DATS ochraňuje endotelové buňky před oxidativním stresem tím, že snižuje hladiny ROS, zvyšuje aktivitu superoxid dismutázy a glutation peroxidázy v mitochondriích a zlepšuje mitochondriální funkce endotelových buněk (Liu *et al.*, 2014).

Antiproliferativní účinky česnekových derivátů jsou zkoumány u nádorových buněk. Alliin indukuje apoptózu nádorových buněk prsu u žen (Izdebska *et al.*, 2016). Alliin zvyšuje expresi tumor-supresorového genu p53 a potlačuje expresi fibroblastového růstového faktoru-2 a potlačuje tak růst lidského fibrosarkomu a nádoru tlustého střeva (Mousa *et* Mousa, 2005). Allicin působí antiproliferativně na lidské nádorové buňky prsou, endometria a tlustého střeva. Tato aktivita allicinu je zprostředkována jeho schopností snížit hladiny glutationu v nádorových buňkách (Hirsch *et al.*, 2000). Allicin způsobuje nárůst exprese kaspázy-3, fragmentaci DNA, zastavení buněčného cyklu, produkci ROS a vyčerpání glutationu v nádorových buňkách pankreatu a způsobuje tak jejich apoptózu (Chhabria *et al.*, 2015). Allicin způsobuje apoptózu lidských epitelových nádorových buněk žaludku. Allicin zde indukuje fragmentaci DNA, přesun proapoptotického faktoru Bax do mitochondrií a uvolnění cytochromu C z mitochondrií do cytosolu (Park *et al.*, 2005).
DADS zvyšuje produkci ROS a aktivuje signální dráhu JNK, čímž spouští apoptózu buněk neuroblastomu (Filomeni *et al.*, 2003). DADS zvyšuje expresi proapoptotických faktorů a snižuje expresi antiapoptotických faktorů v nádorových buňkách prsu (Nakagawa *et al.*, 2001). DADS zvyšuje intracelulární hladiny Ca^{2+}, čímž dochází ke zvýšení produkce peroxidu vodíku a aktivaci kaspáz v lidských leukemických buňkách (Park *et al.*, 2002). Česnekové sulfidy indukují apoptózu lidských nádorových buněk prostaty, přičemž DATS je

účinnější než DAS a DADS. DATS hyperfosforyluje a tím inaktivuje antiapoptotické faktory prostřednictvím aktivace JNK a extracelulárním signálem regulované kinázy (Xiao *et al.*, 2004). DATS způsobuje konformační změny proapoptotických faktorů, které vedou k jejich přesunu do mitochondrií (Kim *et al.*, 2007).

Ve srovnání s alliinem a z něj odvozených česnekových derivátů mají sirné sloučeniny obsažené v AGE vyšší a stálejší protektivní účinky v organismu a jsou tak pro organismus bezpečnější (Corzo-Martinez *et al.*, 2007). Každodenní konzumace AGE v dávce 1,8 – 10 g prokazatelně zvyšuje imunitu testovaných osob, přičemž ani nejvyšší použité koncentrace nevyvolávají žádné vedlejší nežádoucí účinky (Amagase, 2006).

AGE představuje produkt úpravy česneku s nejvyšší antioxidativní aktivitou. Antioxidativní účinek AGE je zajištěn jeho schopností vychytávat volné radikály (Imai *et al.*, 1994) a zvyšovat hladiny enzymů s antioxidativní aktivitou (Geng *et* Lau, 1997). AGE ochraňuje molekuly LDL před oxidací (Horie *et al.*, 1989), brání poškození buněčných membrán a peroxidaci lipidů v plicních endoteliálních buňkách vystavených oxidované formě LDL (Ide *et al.*, 1997) a snižuje produkci peroxidu vodíku a superoxidového aniontu. AGE zvyšuje hladiny glutationu a aktivitu superoxid dismutázy, glutation peroxidázy a glutation reduktázy v buňkách endotelu (Yamasaki *et al.*, 1994; Wei *et* Lau, 1998).

SAC je hlavní složkou AGE. Je pravděpodobné, že za největší část antioxidativních účinků česneku je zodpovědný právě SAC. Kultivace kardiomyocytů s extraktem připraveným z česnekové slupky a dřeně snižuje oxidativní stres a brání hypertrofii a apoptóze. Česneková slupka nicméně neobsahuje alliin ani allicin (Louis *et al.*, 2012). Naproti tomu obsahuje SAC, což nasvědčuje jeho schopnosti snižovat oxidativní stres a ochraňovat buňky před apoptózou v nezávislosti na přítomnosti jiných sirných sloučenin. Tento fakt byl prokázán také ošetřením nervové tkáně extraktem z česnekové slupky po navození ischémie a následném poškození tkáně reperfuzí. SAC obsažený v česnekové slupce pozitivně ovlivňoval přežitelnost neuronů a zlepšoval mitochondriální funkce postižených buněk, bránil redukci potenciálu na mitochondriální membráně a syntéze ATP (Cervantes *et al.*, 2013).

Mechanismus účinku česnekových derivátů prostřednictvím uvolněného sulfanu

Účinky česnekových derivátů jsou v řadě typů tkání zprostředkovány jejich schopností zasahovat do signálních drah produkce gasotransmiteru sulfanu. Česnek ochraňuje kardiomyocyty před oxidativním stresem, hypertrofií a apoptózou tím, že zvyšuje produkci tohoto gasotransmiteru (Louis *et al.*, 2012). Česnek ochraňuje kardiomyocyty prostřednictvím

uvolněného sulfanu před ischemicko-reperfúzním poškozením změnou poměru proapoptotických a antiapoptotických faktorů Bax a Bcl-2 a snížením fosforylace a tím aktivity MAPK a JNK, kináz, které se uplatňují v aktivaci apoptotické signální dráhy (Mukherjee *et al.*, 2009).

DATS ochraňuje kardiomyocyty diabetických potkanů před oxidativním stresem a apoptózou prostřednictvím zvýšení aktivity enzymu CSE a následné zvýšené produkce sulfanu (Tsai *et al.*, 2015). DATS zvyšuje expresi enzymů CBS a CSE a tak produkci endogenního sulfanu v lidských jaterních buňkách a ochraňuje je tak před oxidativním stresem a apoptózou (Chen *et al.*, 2016).

Ošetření SAC snížilo úmrtnost a poškození srdce při uměle vyvolaném infarktu u potkanů. Zablokování aktivity CSE potlačilo tento pozitivní efekt SAC. SAC zde způsobil přímé zvýšení aktivity CSE, ale sloužil také jako substrát pro CSE, která ho přeměnila na sulfan (Chuah *et al.*, 2007).

2.5 *In vitro* kultivace oocytů

Procesy meiotického zrání a oplození oocytu není možné studovat bez využití kultivace oocytů v *in vitro* podmínkách. *In vitro* kultivace oocytů s sebou nesou riziko variability řady faktorů stěžejních pro úspěšný průběh procesů meiotického zrání a oplození, jako jsou složení kultivačního média, pH, úroveň osvětlení (Takenaka *et al.*, 2007) a hladiny kyslíku (Agarwal *et al.*, 2003). Kultivační média jsou doplňována řadou látek, jako jsou séra (Esfandiari *et al.*, 2005), energetické zdroje (Hashimoto *et al.*, 2000) a homony (Markides *et al.*, 1998), které mohou narušit rovnováhu reaktivních forem kyslíku (ROS – reactive oxygen species).

Kultivační média pro *in vitro* kultivace oocytů jsou jednoduché roztoky solí. Jako základ kultivačních médií pro prasečí oocyty je používán Earleho roztok solí obsahující chlorid vápenatý, chlorid sodný, chlorid draselný, dihydrogenfosforečnan sodný a síran hořečnatý.

Jako gonadotropní hormony jsou aplikovány sérový gonadotropin březích klisen a lidský choriový gonadotropin, jejichž účinky odpovídají účinkům hypofyzárních gonadotropinů FSH a LH. FSH zlepšuje kvalitu folikulárních buněk (Hillier *et al.*, 1995). Při aplikaci FSH je důležité dodržení správné koncentrace. Zatímco dávka 10^{-1} I.U./ml narušuje komunikaci oocytu a kumulárních buněk, dávka 10^{-4} I.U./ml zachovává správnou funkci spojení typu *gap junctions* u skotu (Luciano *et al.*, 2011). Také steroidní hormony sehrávají důležitou úlohu během meiotického zrání oocytů. Nedostatek estradiolu během počáteční fáze zrání způsobuje pozdější narušení časného embryonálního vývoje (Mattioli *et al.*, 1998b).

Růstové faktory, mezi které patří inzulinu podobný růstový faktor I a epidermální růstový faktor, způsobují post-translační modifikace estrogenového receptoru a regulují tak jeho funkci (Ignar-Trowbridge *et al.*, 1996). Epidermální růstový faktor stimuluje mitózu a působí tak pozitivně na vystoupení oocytu z prvního meiotického bloku (Lindbloom *et al.*, 2008). Kombinace epidermálního růstového faktoru a FSH působí pozitivně na jaderné i cytoplazmatické zrání oocytů u ovcí (Funahashi *et al.*, 2008). Také u prasete byl prokázán synergistický účinek epidermálního růstového faktoru s FSH na cytoplazmatické zrání (Kwak *et al.*, 2012).

Jako zdroje bílkovin jsou do kultivačních médií aplikována séra, jejichž přítomnost v kultivačním médiu zlepšuje kvalitu oocytů. Fetální telecí sérum představuje nejčastěji aplikované sérum v *in vitro* kultivačních systémech pro oocyty (Sutton *et al.*, 2003). Séra obsahují nedefinovanou směs bílkovin, lipidů, hormonů a růstových faktorů, a jsou proto v některých laboratořích nahrazována definovanou směsí bílkovin nebo bovinním sérovým albuminem, který vykazuje nižší variabilitu složení (Sutton *et al.*, 2003; Dal Canto *et al.*, 2012). Bovinní sérový albumin na sebe váže steroidy, zejména estradiol, a to v množství dostačujícím pro úspěšný průběh jaderného a cytoplazmatického zrání (Mingoti *et al.*, 2002).

Jako zdroj energie pro zrající oocyty jsou aplikovány zejména sacharidy. Přítomnost glukózy je stěžejní při znovuzahájení meiózy u prasat. Současné doplnění kultivačního média glukózou a pyruvátem sodným působí pozitivně na cytoplazmatické zrání prasečích oocytů (Wu *et al.*, 2011). Laktát vápenatý působí pozitivně na mitochondriální funkce a pomáhá tak udržet dostatečnou produkci ATP (Das *et al.*, 1991). Dalším sacharidem využívaným jako zdroj energie je pyruvát sodný (Moschini *et al.*, 2011). Zdrojem energie mohou být také aminokyseliny, například glutamin.

Oocyty jsou kultivovány v podmínkách řízené atmosféry ve směsi 5% CO_2 v atmosférickém vzduchu. Oxid uhličitý slouží k udržení stálého pH, je zdrojem uhlíku pro syntézu nukleových kyselin a proteinů. Někteří autoři navrhují snížení hladiny kyslíku z 20 % v atmosférickém vzduchu na 5 %, tedy hodnotě bližší fyziologickým podmínkám, protože vysoké koncentrace kyslíku způsobují nadměrnou produkci ROS v oocytech (Banwell *et al.*, 2007; Guo *et al.*, 2014).

Vyvážené hladiny ROS jsou nezbytné pro úspěšný průběh meiotického zrání oocytu. ROS jsou endogenně produkovány aktivitou enzymu NADPH oxidázy v buněčných membránách, mitochondriích, peroxizómech a endoplazmatickém retikulu (Nasr-Esfahani *et* Johnson, 1991). Patří mezi ně například peroxid vodíku a superoxidový a hydroxylový radikál. Endogenní antioxidanty brání poškození biologických struktur kyslíkovými radikály.

Redukovaná forma glutationu, superoxid dismutáza, kataláza a glutation peroxidáza jsou nejdůležitějšími endogenními antioxidanty (Banerjee *et al.*, 2003). Na snížení hladin ROS v organismu se podílejí také neenzymatické antioxidanty jako vitamíny A, C a E, pyruvát, glutation, ubiquinon, taurin a hypotaurin (Winyard *et al*., 2005). Rozvoj oxidativního stresu je způsoben nerovnováhou mezi hladinami ROS a endogenní antioxidační kapacitou buněk. Oxidativní stres v oocytech zvyšuje pravděpodobnost vzniku aneuploidie během meiózy (Tarin *et al*., 1998; Hassold *et* Hunt, 2001) a negativně ovlivňuje funkci mikrotubulů dělícího vřeténka (Zuelke *et al*., 1997; Choi *et al*., 2007). Oxidativní stres narušuje homeostázu Ca^{2+}, narušuje redistribuci kortikálních granul, což zvyšuje pravděpodobnost polyspermního oplození (Jiao *et al*., 2013) a snižuje podíl formovaných prvojader v prasečích oocytech po IVF (Alvarez *et al.*, 2015).

Vhodné nastavení *in vitro* kultivačních podmínek je významným faktorem, který ovlivňuje kvalitu oocytů.

3 Hypotézy a cíle

Byla stanovena hypotéza, že kvalitu prasečích oocytů kultivovaných v *in vitro* kultivačním systému lze zlepšit suplementací kultivačního média donorem sulfanu a česnekovými deriváty.

Pro potvrzení formulované hypotézy byly stanoveny cíle:

1. Vyhodnotit vliv suplementace kultivačního média donorem sulfanu během meiotického zrání prasečích oocytů, a to zhodnocením jadrného a cytoplazmatického zrání, kumulární expanze a časného embryonálního vývoje kultivovaných oocytů. Dílčími cíly bylo:
    - vyhodnotit vliv donoru sulfanu na jaderné zrání morfologickým hodnocením oocytů,
    - vyhodnotit vliv donoru sulfanu na cytoplazmatické zrání prostřednictvím hodnocení aktivity MPF a MAPK,
    - vyhodnotit vliv donoru sulfanu na kumulární expanzi prostřednictvím hodnocení produkce hyaluronové kyseliny kumulo-oocytárními a oocytektomovanými komplexy,
    - vyhodnotit vliv suplementace média donorem sulfanu během meiotického zrání oocytů na jejich časný embryonální vývoj.

2. Vyhodnotit vliv suplementace kultivačního média donorem sulfanu na kvalitu stárnoucích prasečích oocytů během jejich prodloužené kultivace. Dílčími cíli bylo:
    - vyhodnotit endogenní produkci sulfanu ve stárnoucích oocytech,
    - vyhodnotit vliv donoru sulfanu na průběh stárnutí oocytů jejich morfologickým hodnocením,
    - vyhodnotit vliv inhibitorů sulfan uvolňujících enzymů na průběh stárnutí oocytů,
    - vyhodnotit vliv donoru sulfanu na průběh stárnutí oocytů ošetřených inhibitory sulfan uvolňujících enzymů,
    - vyhodnotit vliv donoru sulfanu a inhibitorů sulfan uvolňujících enzymů na aktivitu MPF a MAPK během procesu stárnutí oocytů,
    - vyhodnotit vliv kultivace stárnoucích oocytů s donorem sulfanu na časný embryonální vývoj.

3. Vyhodnotit vliv suplementace kultivačního média česnekovými deriváty během meiotické zrání prasečích oocytů, a to zhodnocením jadrného a cytoplazmatického zrání, kumulární expanze a časného embryonálního vývoje kultivovaných oocytů. Dílčími cíli bylo:
    - vyhodnotit vliv česnekových derivátů na jaderné zrání morfologickým hodnocením oocytů,
    - vyhodnotit vliv česnekových derivátů na cytoplazmatické zrání prostřednictvím hodnocení aktivity MPF a MAPK,
    - vyhodnotit vliv česnekových derivátů na kumulární expanzi prostřednictvím hodnocení produkce hyaluronové kyseliny,
    - vyhodnotit vliv suplementace média česnekovými deriváty během meiotického zrání oocytů na jejich časný embryonální vývoj.

4 Materiál a metody

4.1 Zisk materiálu

Kumulo-oocytární komplexy (COCs) byly získávány z vaječníků prepubertálních prasniček aspirací ovariálních folikulů o průměru 2 - 5 mm pomocí injekční stříkačky a jehly 20G. Pro kultivaci byly vybírány pouze COCs skládající se z plně dorostlých oocytů o průměru 120 μm a kompaktní vrstvy kumulárních buněk.

4.2 Kultivační podmínky

Kultivace kumulo-oocytárních komplexů

COCs byly kultivovány v modifikovaném kultivačním médiu M199 (Sigma-Aldrich, USA) obsahujícím hydrogenuhličitan sodný (32,5 mM), laktát vápenatý (2,75 mM), gentamicin (0,025 mg/ml), HEPES (6,3 mM), bovinní sérový albumin (0,5 g/ml), gonadotropní hormony eCG a hCG v poměru 13,5 I.U.: 6,6 I.U./ml (P.G.600; Intervet, Holandsko) a 10 % (v/v) fetálního bovinního séra (GibcoBRL; Life Technologies, Německo) (5 % CO_2, 39 °C).

Experimentální skupiny byly ošetřeny donorem sulfanu, inhibitory sulfan uvolňujících enzymů a česnekovými deriváty. Jako donor sulfanu byl použit $Na_2S.9H_2O$ v koncentracích 0,035; 0,07; 0,15; 0,3; 0,6 a 0,9 mM (Sigma-Aldrich, USA). Jako inhibitor CBS byla použita 1mM kyselina oxamová (OA – oxamic acid) (Sigma-Aldrich, USA), jako inhibitor CSE 1mM beta-kyano-L-alanin (KA – beta-kyano-L-alanine) (Sigma-Aldrich, USA) a jako inhibitor 3-MPST 5mM dihydrát disodné soli kyseliny alfa-ketoglutarové (KGA –alpha-ketoglutaric acid) (Sigma-Aldrich, USA). Inhibitory byly použity samostatně a v těchto kombinacích: OA+KA, OA+KGA, KA+KGA, OA+KA+KGA. Použité česnekové deriváty byly alliin v koncentracích 0,05 a 0,1 mM (Sigma-Aldrich, USA) a S-allyl cystein v koncentracích 0,1; 0,5; 1 a 5 mM (Sigma-Aldrich, USA).

COCs byly kultivovány 24 hodin do stádia metafáze I a 48 hodin do stádia metafáze II. Pro podrobnější hodnocení meiotického zrání byly oocyty kultivovány 0 – 48 hod. a hodnoceny každé dvě hodiny.

Oocytektomie

Pro hodnocení vlivu přítomnosti oocytu na produkci hyaluronové kyseliny během kultivace s donorem sulfanu byla provedena oocytektomie kumulo-oocytárních komplexů. Oocytektomie probíhala v kultivačním médiu pod minerálním olejem za použití mechanického mikromanipulátoru (Narishige, Japonsko). COCs byly imobilizovány fixační

kapilárou a oocyty byly odstraněny pomocí injekční kapiláry za vzniku oocytektomovaných komplexů (OOXs – oocytectomised complexes). OOXs byly kultivovány za stejných podmínek jako COCs.

Kultivace stárnoucích oocytů

Oocyty ve stádiu metafáze II byly zbaveny kumulárních buněk pomocí opakovaného nasávání tenkostěnnou kapilárou a vystaveny prodloužené kultivaci v modifikovaném médiu M199 ve výše popsaných kultivačních podmínkách bez přídavku hormonů po dobu 24, 48 a 72 hodin.

4.3 Hodnocení meiotického zrání a stárnutí oocytů

Morfologické hodnocení jaderného zrání a stárnutí oocytů

Oocyty byly po dokončení kultivace zbaveny kumulárních buněk pomocí opakovaného nasávání tenkostěnnou kapilárou, montovány mezi podložní a krycí sklo a fixovány v octové kyselině a ethanolu (1:3, v/v) po dobu min. 48 hod. Oocyty byly hodnoceny pod mikroskopem s fázovým kontrastem po obarvení 1% roztokem orceinu.

Jaderné zrání bylo hodnoceno podle kritérií publikovaných Motlíkem a Fulkou (1976) a oocyty byly řazeny do následujících kategorií: GV – zárodečný váček (germinal vesicle); LD – pozdní diakineze (late diakinesis); MI – metafáze prvního meiotického dělení; AITI – přechod z anafáze do telofáze prvního meiotického dělení; MII – metafáze 2. meiotického dělení.

Při morfologickém hodnocení stárnutí oocytů byly oocyty řazeny do následujících kategorií: intaktní oocyty – oocyty ve stádiu MII a AITI; aktivované oocyty – oocyty s formovanými prvojádry a embrya; fragmentované oocyty – oocyty s apoptotickými váčky pod *zonou pellucidou*; lytické oocyty – oocyty s porušenou cytoplazmatickou membránou a změnami odpovídajícími lytickému zániku oocytů.

Hodnocení kinázové aktivity ve zrajících a stárnoucích oocytech

Kinázová esej byla provedena dle Kubelky *et al.* (2000). Vzorky byly připraveny přidáním 5 µl extrakčního pufru a ihned zamrazeny (-80 °C). Specifické substráty MPF a MAPK, histon H1 (H1) a myelinový bazický protein (MBP), byly fosforylovány radioaktivně značeným izotopem 500 µCi/ml [γ-^{32}P]ATP (GE Healthcare Life Sciences, USA).

H1 a MBP byly separovány pomocí SDS-polyakrylamidové elekroforézy na 10% separačním gelu. Intenzita signálu fosforylovaných H1 a MBP byla odečtena pomocí scanaru FLA 7000 reader (GE Healthcare Life Sciences, USA) a softwaru Multi-Gauge 2.0 (Fujifilm, Japonsko).

Hodnocení produkce hyaluronové kyseliny

Po ukončení kultivace bylo kultivační médium s kumulárními buňkami přeneseno do zkumavek Eppendorf a centrifugováno (10 000 rpm; 10 min.). Byly připraveny vzorky z buněčných pelet pro hodnocení produkce HA zadržené COCs a OOXs a vzorky z kultivačního média pro hodnocení celkové produkce HA. Buněčné pelety byly vystaveny proteolytické digesci 30 µl alkalázy 2.4 L FG v PBS (1:100 v/v, Novozymes, Dánsko; 2 hod.) a 30 µl alkalázy Flavourzyme 1000 L (1:100 v/v, Novozymes, Dánsko; 3 hod.). Reakce byla ukončena varem (3 min.). Množství HA ve vzorcích bylo hodnoceno pomocí QnE Hyaluronic Acid ELISA Assay detection kit (Biotech, USA) a stanoveno spektrofotometricky v mikrotitračních destičkách za použití přístroje Rainbow ELISA plate reader (540 nm).

V průběhu řešení této práce byla na pracovišti KVD vyvinuta spektrofotometrická metoda měření produkce HA. Po ukončení kultivace byly COCs 4x opláchnuty ve 450 µl PBS-PVA ve vícejamkových destičkách 4-well multidish (Nunc, Denmark) přenášením pomocí 50µl automatické pipety. Oocyty byly odstraněny ze vzorku a 500 µl PBS-PVA s kumuly bylo přeneseno do zkumavky Eppendorf. Vzorky byly vystaveny digesci lyázou ze *Streptomyces hyaluronoticus* (20 µl/ml; Sigma-Aldrich, USA) při 39 °C přes noc, centrifugovány (10 000 rpm, 5 min., 4 °C) a hodnoceny na spektrofotometru Helios Epsilon (Verkon, ČR; 216 nm).

4.4 Hodnocení endogenní produkce sulfanu

Endogenní produkce sulfanu byla hodnocena v oocytech ve stádiu metafáze II a ve stárnoucích oocytech po 24, 48 a 72 hod. prodloužené kultivace. Oocyty byly mechanicky rozrušeny v reakční směsi 5 µl pyridoxal-5-fosfátu (Sigma-Aldrich, USA; 0,2 M) a 50 µl L-cysteinu (Sigma-Aldrich, USA; 10 mM) ve 445 µl redestilované vody (4 °C). Dále byl přidán acetát zinku (Sigma-Aldrich, USA; 1%, 250 µl). Enzymatická reakce probíhala pod tekutým N_2 (60 min.; 37 °C) a ukončena byla přidáním 50% trichloroctové kyseliny (Sigma-Aldrich, USA; 250 µl). Reakční směs byla dále inkubována (60 min.; 37 °C). Poté byl přidán N,N-dimetyl-p-fenylendiamin sulfát (Sigma-Aldrich, USA; 20 mM v 7,2M HCl; 133 µl) a $FeCl_3$ (Sigma-Aldrich, USA; 30 mM v 1,2M HCl; 133 µl). Vzorky byly měřeny na spektrofotometru (670 nm).

4.5 Hodnocení produkce reaktivních forem kyslíku

Produkce reaktivních forem kyslíku byla hodnocena v oocytech po 24 a 48 hodinách meiotického zrání a v zygotách 22 hodin po partenogenetické aktivaci. Oocyty a zygoty byly

3x opláchnuty v PBS-PVA, inkubovány v PBS-PVA s 2′,7′-dichlorofluorescin diacetátem (Sigma-Aldrich, USA; 10 μM; 20 min., 39 °C), 3x opláchnuty v PBS-PVA a montovány mezi podložní a krycí sklo. Preparáty byly hodnoceny na konfokálním mikroskopu (Leica SPE, Německo; exc. 450 - 490 nm), intenzita signálu byla měřena pomocí analýzy obrazu NIS Elements 4.0.

4.6 Partenogenetická aktivace a hodnocení časného embryonálního vývoje

Po skončení meiotického zrání byly oocyty zbaveny kumulárních buněk a aktivovány ionforem vápníku A23187 (Sigma-Aldrich, USA; 25 μM, 5 min.) a následně kultivovány s 6-dimetylaminopurinem (Sigma-Aldrich, USA; 2 mM, 2 hod.).

V experimentech hodnotících vliv kultivace se sulfanem na časný embryonální vývoj byly aktivované oocyty kultivovány v médiu NCSU23 (39 °C, 5 % CO_2). Časný embryonální vývoj byl hodnocen pod mikroskopem s fázovým kontrastem. Aktivační potenciál byl hodnocen na základě formace prvojader 24 hodin po partenogenetické aktivaci. Rýhování bylo hodnoceno 48 hodin po partenogenetické aktivaci. Formace morul a blastocyst byla hodnocena sedm dní po partenogenetické aktivaci.

V experimentech hodnotících vliv kultivace s česnekovými deriváty na časný embryonální vývoj byly aktivované oocyty kultivovány v modifikovaném médiu M199 bez hormonů (39 °C, 5 % CO_2). Časný embryonální vývoj byl hodnocen pod mikroskopem s fázovým kontrastem. Aktivační potenciál a časné rýhování byly hodnoceny 22 hodin po partenogenetické aktivaci.

4.7 Statistická analýza

Každý experiment byl opakován nejméně čtyřikrát. Výsledky experimentů byly podrobeny statistické analýze v programu SAS (SAS Institute Inc., USA) za použití testu ANOVA. P hodnota menší než 0,05 byla považována za statisticky významnou.

5 Publikované práce

5.1 Vliv suplementace kultivačního média donorem sulfanu během meiotického zrání prasečích oocytů

Endogenně produkovaný sulfan je jedním z faktorů, které ovlivňují průběh meiotického zrání oocytů. Stanovená hypotéza, že suplementace kultivačního média donorem sulfanu může zvýšit kvalitu oocytů kultivovaných v *in vitro* podmínkách, byla ověřena prostřednictvím Na_2S, který byl v koncentraci 0,3 mM přidáván do kultivačního média. Suplementace kultivačního média donorem sulfanu urychlila průběh meiotického zrání kultivovaných oocytů a zlepšila jejich časný embryonální vývoj.

Vliv donoru sulfanu na jaderné zrání byl hodnocen morfologickým hodnocením zrajících oocytů. Suplementace média Na_2S zapříčinila nárůst podílu oocytů, které po 20 hodinách kultivace prodělaly GVBD (80 % vs. 68,3 %). Vzrostl i podíl oocytů, které prošly přechodem z meiózy I do meiózy II po 30 hodinách kultivace (86,7 % vs. 57,5 %). Přítomnost Na_2S v kultivačním médiu zároveň urychlila nárůst aktivity MPF a MAPK časově korespondující s nástupem GVBD, tedy po 20 - 22 hodinách kultivace.

Donor sulfanu potlačil celkovou produkci hyaluronové kyseliny kumulo-oocytárními komplexy po 36 hodinách kultivace o 13 % a po 48 hodinách kultivace o 29 %, avšak rozdíl mezi kontrolní a pokusnou skupinou byl zjištěn pouze u kumulo-oocytárních komplexů. Oocytektomie sice snížila celkovou produkci hyaluronové kyseliny, ale vliv donoru sulfanu na ocytektomované komplexy nebyl prokázán.

Oocyty, které zrály v přítomnosti donoru sulfanu, vykazovaly lepší aktivační potenciál. Po partenogenetické aktivaci bylo aktivováno 91,7 % oocytů v experimentální skupině oproti 75,8 % oocytů v kontrolní skupině. Rýhování a formace morul a blastocyst ale ovlivněny nebyly.

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Dual Effects of Hydrogen Sulfide Donor on Meiosis and Cumulus Expansion of Porcine Cumulus-Oocyte Complexes

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Abstract

Hydrogen sulfide (H_2S) has been revealed to be a signal molecule with second messenger action in the somatic cells of many tissues, including the reproductive tract. The aim of this study was to address how exogenous H_2S acts on the meiotic maturation of porcine oocytes, including key maturation factors such as MPF and MAPK, and cumulus expansion intensity of cumulus-oocyte complexes. We observed that the H_2S donor, Na_2S, accelerated oocyte *in vitro* maturation in a dose-dependent manner, following an increase of MPF activity around germinal vesicle breakdown. Concurrently, the H_2S donor affected cumulus expansion, monitored by hyaluronic acid production. Our results suggest that the H_2S donor influences oocyte maturation and thus also participates in the regulation of cumulus expansion. The exogenous H_2S donor apparently affects key signal pathways of oocyte maturation and cumulus expansion, resulting in faster oocyte maturation with little need of cumulus expansion.

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Introduction

Previously, molecules of some gases have been discovered to have biological activities. These gases, so called gasotransmitters, act as second messengers in the signal transduction of cell communication. In addition to the earlier observed nitric oxide and carbon monoxide, the role of hydrogen sulfide in cell metabolism has recently been studied [1]. Hydrogen sulfide (H_2S) is enzymatically released from aminoacid L-cystein by Cystathionine β-Synthase (CBS), Cystathionine γ-Lyase (CSE) and 3-Mercaptopyruvate Sulfurtransferase (3-MPST) [2–4]. These enzymes are expressed in several tissues, including in the reproductive system [5–7], where it can be assumed that H_2S production mediates physiological functions. The presence and effect of CBS in the ovarian follicles of mice has been determined [8,9]. The role of H_2S in oocyte maturation is not yet clear and has not been unravelled.

Successful meiotic maturation of oocytes is an important precondition of reproductive biotechnological progress. Only fully grown dictyate oocytes in germinal vesicle stage (GV-oocytes) undergo complete meiotic maturation and achieve metaphase II [10]. This process resumes after the hormonal stimuli action of the oocyte reinitiates meiotic division by the activation of key regulatory factors, such as Maturation/M-phase Promoting Factor (MPF) and Mitogen Activated Protein Kinase (MAPK), resulting in germinal vesicle breakdown (GVBD). Activation and correct kinesis of these factors are further necessary for meiosis I to II transition, organisation of the second meiotic metaphase spindle and spontaneous metaphase II-block [11–17]. The cytoplasmic changes of key factors of oocyte maturation are dependent upon intercellular communication between oocyte and surrounding cumulus cells [10]. On the other hand, mucification of the cumulus cells, known as cumulus expansion, causes a decrease of inhibitory substance flows into oocyte, especially cAMP, and restricted input of cAMP allows MPF activation, which triggers GVBD [18].

The cumulus expansion consists of synthesis and accumulation of glycosaminoglycans, especially hyaluronic acid, into the extracellular space [19]. Thus, cumulus expansion expressed by hyaluronic acid content may be a possible marker of successful GVBD, meiotic maturation and developmental competence acquisition in oocytes used for biotechnologies, i.e. *in vitro* fertilisation, transgenesis or cloning [20–23].

Meiotic maturation and cumulus expansion are simultaneously regulated by a complex network of several signal pathways

including cAMP-PKA, Plk1-Cdc25-Cdc2, PI3K-Akt and Mos-MEK-MAPK [24–28]. Noticeably, the PI3K-Akt and cAMP-PKA pathways have been reported to be regulated by H_2S during the cell cycle of somatic cells [29–32]. Full knowledge of the molecular mechanisms of oocyte maturation and H_2S involvement in meiosis could improve the yield of successfully *in vitro* matured oocytes. We hypothesised that H_2S plays a role in the regulation of meiotic oocyte maturation. The aim of this study was to evaluate the influence of the H_2S donor on oocyte maturation, regulatory kinase activity in oocytes and the cumulus expansion intensity of porcine cumulus-oocyte complexes (COCs) cultivated *in vitro*.

For this purpose, we tested the influence of the exogenous H_2S donor, Na_2S, on oocyte maturation, developmental competence acquisition and cumulus expansion of COCs. Here, we report for the first that the H_2S donor acts on oocytes to regulate cumulus expansion and progression through meiosis.

Materials and Methods

In Vitro Oocyte Cultivation with H_2S Donor

Porcine ovaries were obtained from non-cycling gilts at the local slaughterhouse (Jatky Plzen a.s., Plzen, Czech Republic). Ovaries were transported to the laboratory in a saline solution (0.9% NaCl) at 39°C. Cumulus-oocyte complexes (COCs) were collected from ovarian follicles with a diameter of 2 – 5 mm by a 20-gauge aspirating needle. Only fully grown oocytes with intact cytoplasm surrounded by compact cumuli were used in further experiments.

The COCs were matured in a modified M199 medium (Sigma-Aldrich, USA) supplemented with 32.5 mM sodium bicarbonate, 2.75 mM calcium L-lactate, 0.025 mg/ml gentamicin, 6.3 mM HEPES, 13.5 IU eCG: 6.6 IU hCG/ml (P.G.600; Intervet, Holland) and 5% (v/v) fetal bovine serum (Sigma-Aldrich, USA). The culture medium contained 150, 300, 600 or 900 μM $Na_2S.9H_2O$ (Sigma-Aldrich, USA), the H_2S donor. The COCs were matured for 6–48 hs in 3.5 cm Petri dishes (Nunc) containing 3.0 ml of culture medium at 39°C in a mixture of 5.0% CO_2 in air.

Evaluation of Oocyte Meiotic Maturation

At the end of culture, the COCs were treated with 1 mg/ml bovine testicular hyaluronidase (Sigma-Aldrich, USA) dissolved in M199 medium and cumulus cells were separated from oocytes by repeated pipetting through a narrow glass pipette. The oocytes were subsequently mounted on microscope slides with vaseline, covered with a cover glass, and fixed in ethanol-acetic acid (3:1 v/v) for at least 48 h. The oocytes were stained with 1.0% orcein in 50% aqueous-acetic acid and examined under a phase contrast microscope. Five groups of meiotic maturation stages were determined in accordance with the published criteria by Motlik *et* Fulka [33]: GV – germinal vesicle, LD – late diakinesis, MI - metaphase I, AITI – anaphase I to telophase I transition, MII – metaphase II.

Histone H1 and Myelin Basic Protein Double Assay

The COCs were matured for 12 – 48 hs with the H_2S donor. At each time interval during the culture, COCs were denuded and 10 oocytes per sample were collected. Assays were performed in accordance with the protocol of Kubelka *et al.* [34], with slight modifications. Briefly, the oocytes were washed four times in 0.01% polyvinyl alcohol in PBS, and transferred into 5 μl of buffer containing 40 mM 3-[n-morpholino] propanesulfonic acid pH 7.2, 20 mM para-nitrophenyl phosphate, 40 mM β-glycerolphosphate, 10 mM EGTA, 0.2 mM EDTA, 2 mM dithiothreitol, 0.2 mM Na_3VO_4, 2 mM benzamidine, 40 μg/ml leupeptin and 40 μg/ml aprotinin. Samples were immediately frozen and stored in Eppendorf tubes at −80°C until assays were performed. An assay of MPF and MAP kinase activity by their capacity to phosphorylate external substrates, specifically histone H1 (H1) and Myelin Basic Protein (MBP), was performed. The kinase reaction was initiated by addition of 5 μl of buffer consisting of 100 mM 3-[n-morpholino] propanesulfonic acid pH 7.2, 20 mM para-nitrophenyl phosphate, 40 mM β-glycerolphosphate, 20 mM $MgCl_2$, 10 mM EGTA, 0.2 mM EDTA, 5 μM cAMP-dependent protein kinase inhibitor, 2 mM benzamidine, 40 μg/ml leupeptin, 40 μg/ml aprotinin, 600 μM ATP, 2 mg H1/ml, 3 mg MBP/ml) and 500 μCi/ml [γ-^{32}P]ATP (GE Healthcare Life Sciences, UK). The reaction was conducted for 30 min at 30°C and terminated by the addition of 10 μl Laemmli sample buffer and boiling for 3 min. After electrophoresis on 15% SDS PAGE gels, it was stained with Coomasie Blue R250, destained overnight, dried and autoradiographed. Phosphorylated histone H1 and MBP signals were visualised by MultiGauge 2.0 software and related to metaphase I oocytes after 24 h cultivation, where we expected the peak of kinase activity [34].

Oocytectomy and OOXs Cultivation

The COCs obtained using the above-detailed procedure were oocytectomised in accordance with Prochazka *et al.* [35]. Each COC was immobilised with a holding pipette. A glass needle was then introduced through the cumulus cells and the oocyte into the holding pipette, allowing the ooplasm to be sucked into the holding pipette. After withdrawal of the needle, the ooplasm, but not the zona pellucida, was aspirated into the holding pipette by a burst of a negative pressure. The technique was performed in a drop of culture medium covered by mineral oil in a Petri dish. A set of 25 oocytectomised complexes (OOXs) was prepared within 30 min and immediately placed into the culture. The further cultivation of OOXs took place under the already described conditions.

Hyaluronic Acid Assay

Groups of 25 COCs or OOXs were cultured for 12–48 hs in 1 ml culture modified M199 medium. The culture medium with cumulus cells after denuding of oocytes, or with OOXs, was placed into an Eppendorf tube and centrifugated at 10 000 rpm for 10 min. Cell pellets were proteolytically digested by 30 μl Alcalase 2.4 L FG in PBS (1:100 v/v, Novozymes, Denmark) for 2 hs and thereafter 30 μl Flavourzyme 1000 L (1:100 v/v, Novozymes, Denmark) was added and the mixture was cultured for a further 3 hs. The reaction was terminated by boiling for 3 min and the samples were stored at −20°C until the assay was performed. In addition to cell pellet digestion, the aliquots of culture medium for hyaluronic acid measurement were prepared. The HA content was ascertained by enzyme-linked immunosorbent assay. The QnE Hyaluronic Acid ELISA Assay detection kit (Biotech, USA) was used to determine it. The amount of HA was measured spectrophotometrically on a microtitration plate using a Rainbow ELISA plate reader (wavelength 540 nm). The quadratic calibration curve was based on five standard concentrations of HA. Synthesis of HA was expressed either as the total HA production (HA content in cell pellet and medium) or the retained HA (HA content in cell pellet only). For each concentration of H_2S donor and point of time scale, the measured values of total HA were related to the control group of oocytes after 48 h cultivation.

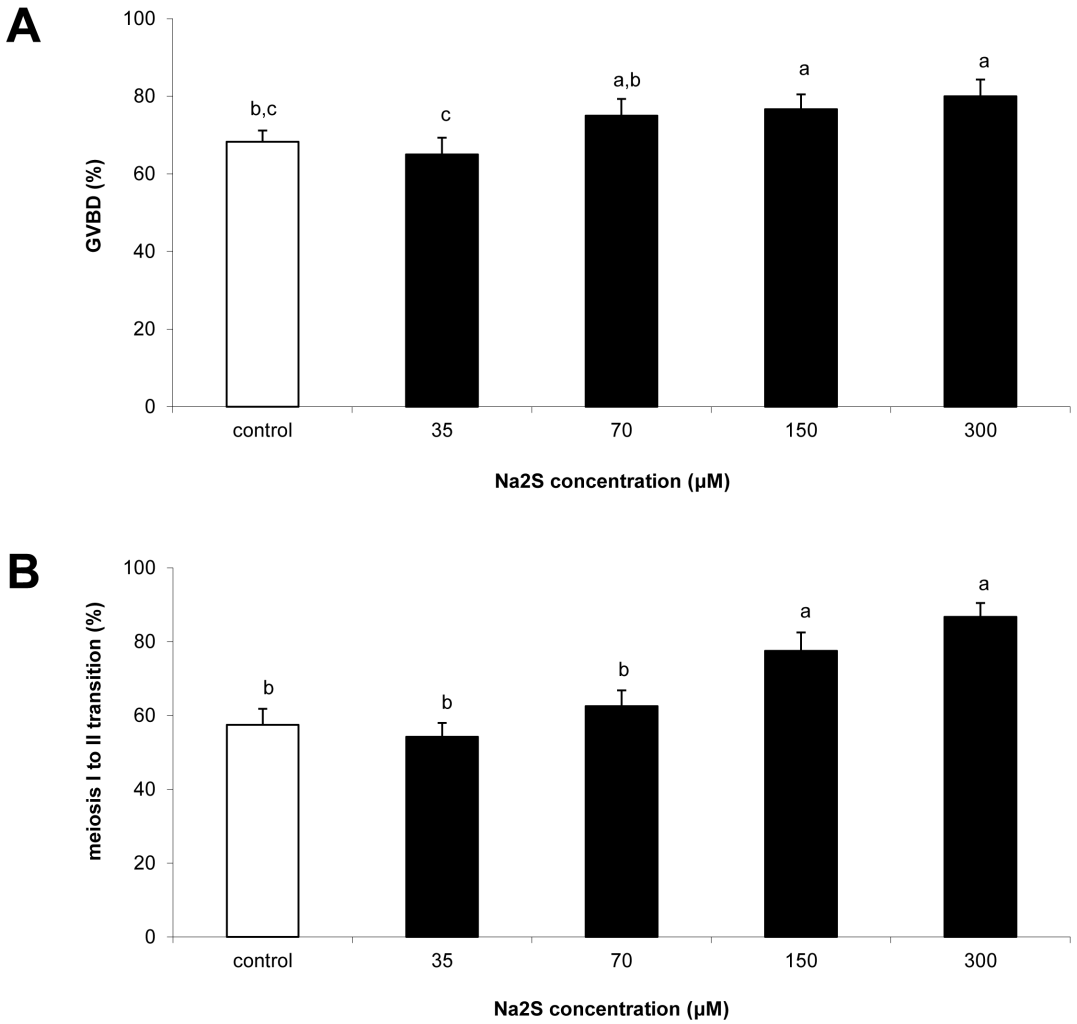

Figure 1. Effect of different Na_2S concentrations on meiosis resumption and transition to meiosis II in oocytes. Proportion of GVBD (A) and meiosis I to II transition (B) during *in vitro* cultivation after 20 and 30 h *in vitro* cultivation, respectively. [a,b,c]Statistically significant differences among experimental groups ($P<0.05$).
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Parthenogenetic Activation of Oocytes

Oocytes were partenogenetically activated using our previously published protocol [36]. Briefly, oocytes were matured *in vitro* for 44 and 46 hs with and without the H_2S donor, respectively. After in vitro maturation, oocytes were denuded and activated for 5 min with 25 μM calcium ionophore A23185. After activation, the oocytes were cultured for 2 hs with 2 mM 6-dimethylaminopurine (DMAP) in NCSU23 medium [37]. The oocytes were then cultured for 24 hs or 7 days in four-well Petri dishes (Nunc) containing 1.0 ml of culture medium under described conditions. Subsequently, oocytes were fixed and stained as described above. Oocytes with pronuclei were considered to be activated. In a separate experiment after oocyte activation, the presumptive zygotes were cultured for 7 days. The cleavage rate and blastocyst achievement was assessed after 2 and 7 days of culture, respectively.

Statistical Analysis

Our data are from at least three independent experiments. The general linear models (GLM) procedure in SAS software (SAS Institute Inc., USA) was used to analyse data from all experiments. Significant differences between groups were determined using the t-test. The level of significance was set at $P<0.05$.

Results

H_2S Donor Accelerates Oocyte Maturation in a Dose-Dependent Manner

We evaluated the influence of different concentrations of H_2S donor on the nuclear maturation of porcine oocytes after 20 and 30 hs of *in vitro* cultivation. Time points of 20 and 30 hs were selected to represent more meiotic stages.

No effect of the H_2S donor Na_2S for the lowest concentration of 35 μM was observed after 20 and 30 h cultivation. With increasing concentration of Na_2S accelerating GVBD (75.0–80.0 vs. 68.3% for H_2S donor and control, respectively) after 20 h cultivation, the differences were statistically significant (Figure 1A, Table S1a). With higher concentration of the H_2S donor, acceleration of meiosis I to II transition in oocytes was observed after 30 h cultivation (Figure 1B). As such, these oocytes achieved meiosis II with statistical differences in 77.5

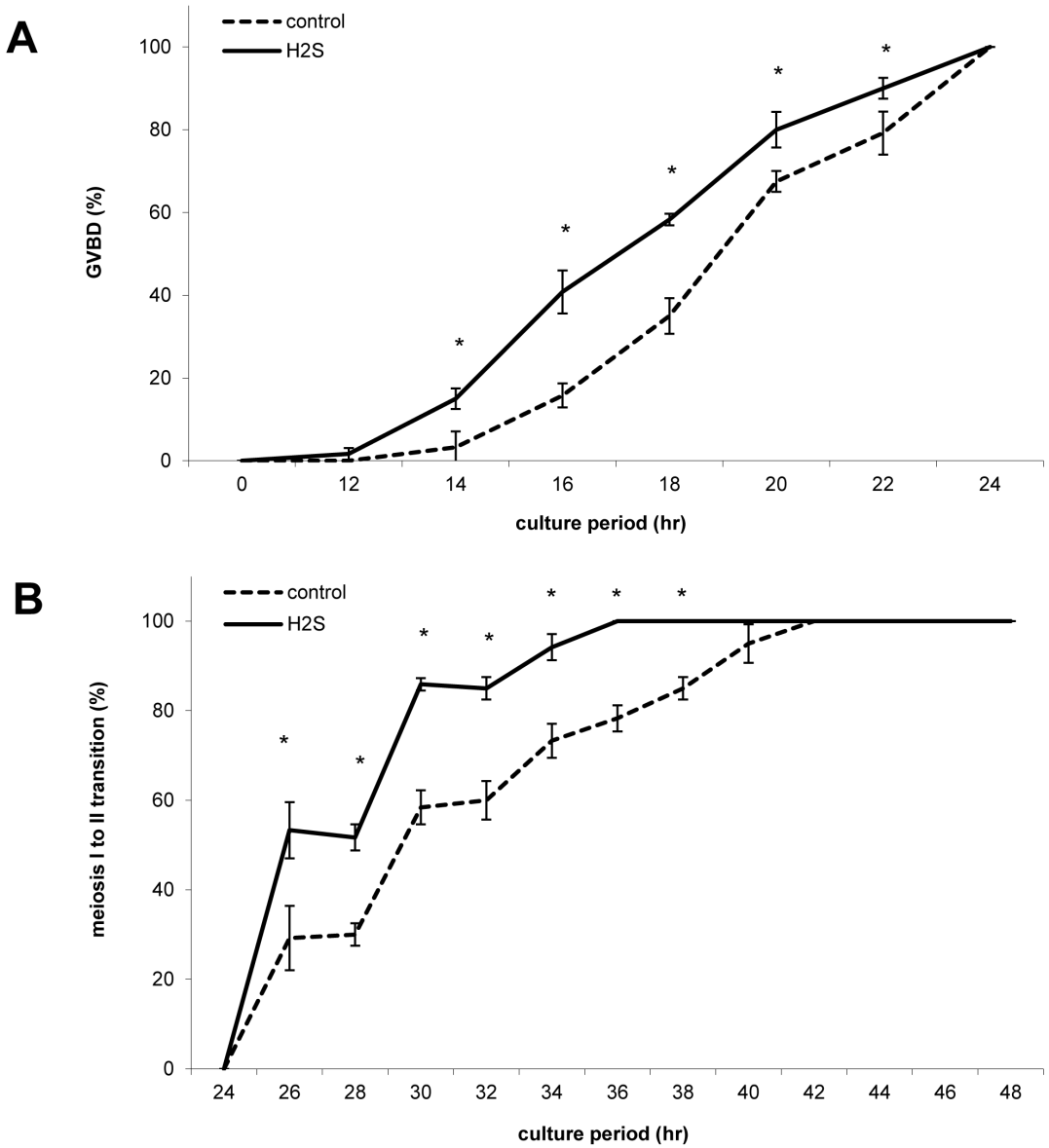

Figure 2. Effect of Na_2S on meiotic resumption and transition to meiosis II during oocyte cultivation. Proportion of GVBD (A) and meiosis I to II transition (B) in oocytes during *in vitro* cultivation over 2 h time scale. H_2S: 300 μM Na_2S. *Statistically significant differences between control and H_2S groups ($P<0.05$).
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and 86.7% of cases for 150 and 300 μM Na_2S, respectively (see more in Table S1b).

H_2S Donor Accelerates Porcine Oocyte Maturation

We evaluated the influence of H_2S donor Na_2S on nuclear maturation of porcine oocytes during *in vitro* cultivation over a 2 h time scale. We monitored the effect of 300 μM Na_2S on germinal vesicle breakdown (GVBD). An accelerated decline of the amount of germinal vesicle (GV) oocyte together with GVBD increase were statistically significant after 14–20 h cultivation (Figure 2A). Moreover, H_2S donor-treated oocytes reached faster meiosis II than the control ones (Figure 2B). The complete data are provided in Table S2.

MPF and MAPK Activity Profiles Are Accelerated by H_2S Donor

To further characterise the effect of H_2S on oocyte maturation, a kinase activity assay was performed (Figure 3A, 3B, Figure S1). We observed the influence of H_2S donor, Na_2S, in 300 μM concentration on the beginning of MPF and MAPK activity around GVBD over a 2 h time scale. Data were expressed relative to MPF/MAPK activity in oocytes cultivated for 24 h where it is predictable that kinase activity is the highest. The phosphorylated histone H1 and MBP signal intensities reflecting the MPF and MAPK activity profile, respectively, were increased and accelerated by the H_2S donor during oocyte maturation. The difference in MAPK activity between the control and H_2S groups was statistically significant after 20 h *in vitro* cultivation. During further

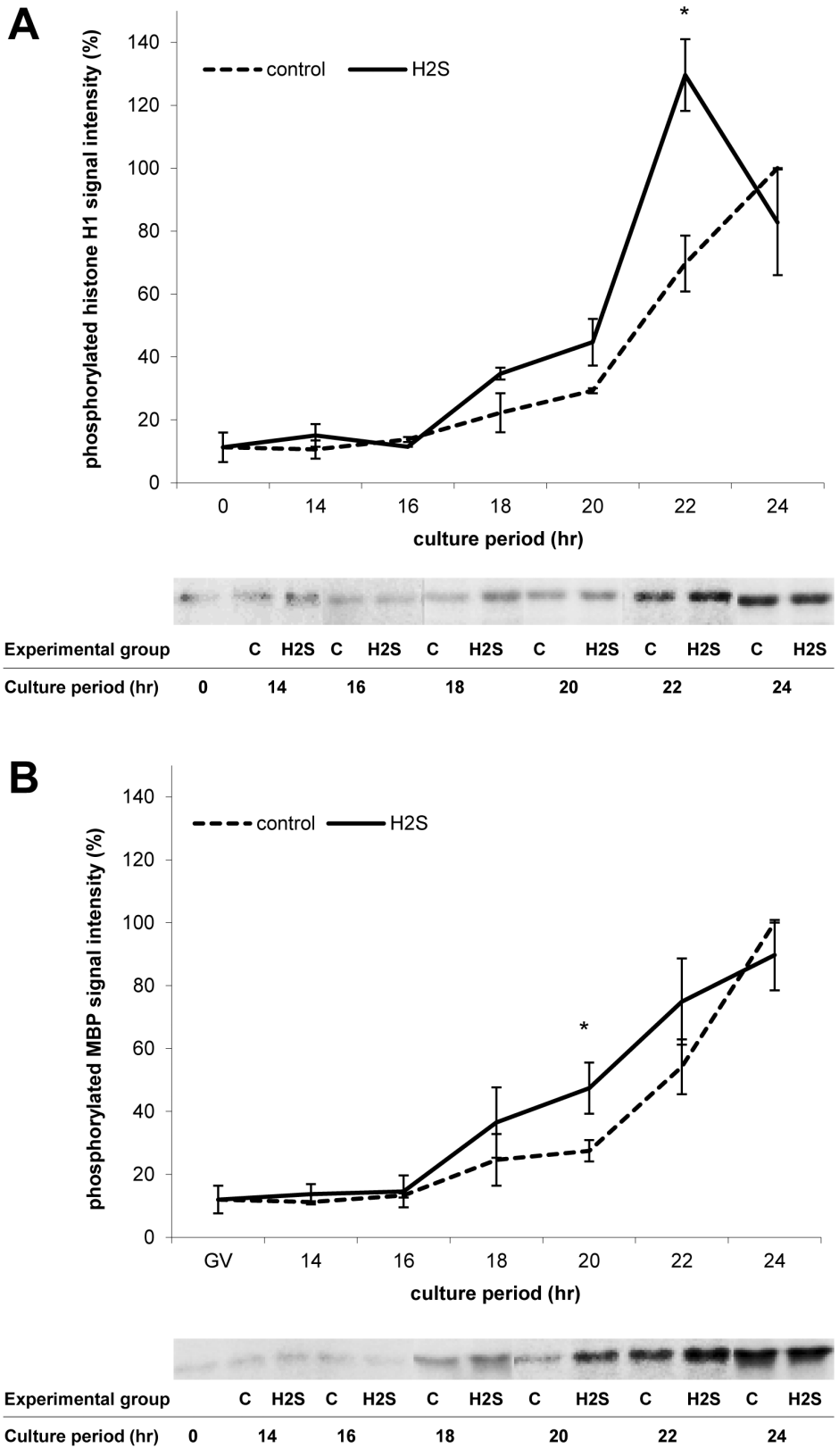

Figure 3. Effect of Na_2S on MPF and MAPK activities during oocyte cultivation. Representative autoradiograms and signal quantifications of phosphorylated histone H1 (A) and MBP (B) reflecting MPF and MAPK activity, respectively. Kinase activity was measured in oocytes cultivated with or without Na_2S over 2 h time scale. The kinase activity was related to oocytes cultivated for 24 hs. C: control; H_2S: 300 μM Na_2S. *Statistically significant differences between control and H_2S groups ($P<0.05$).
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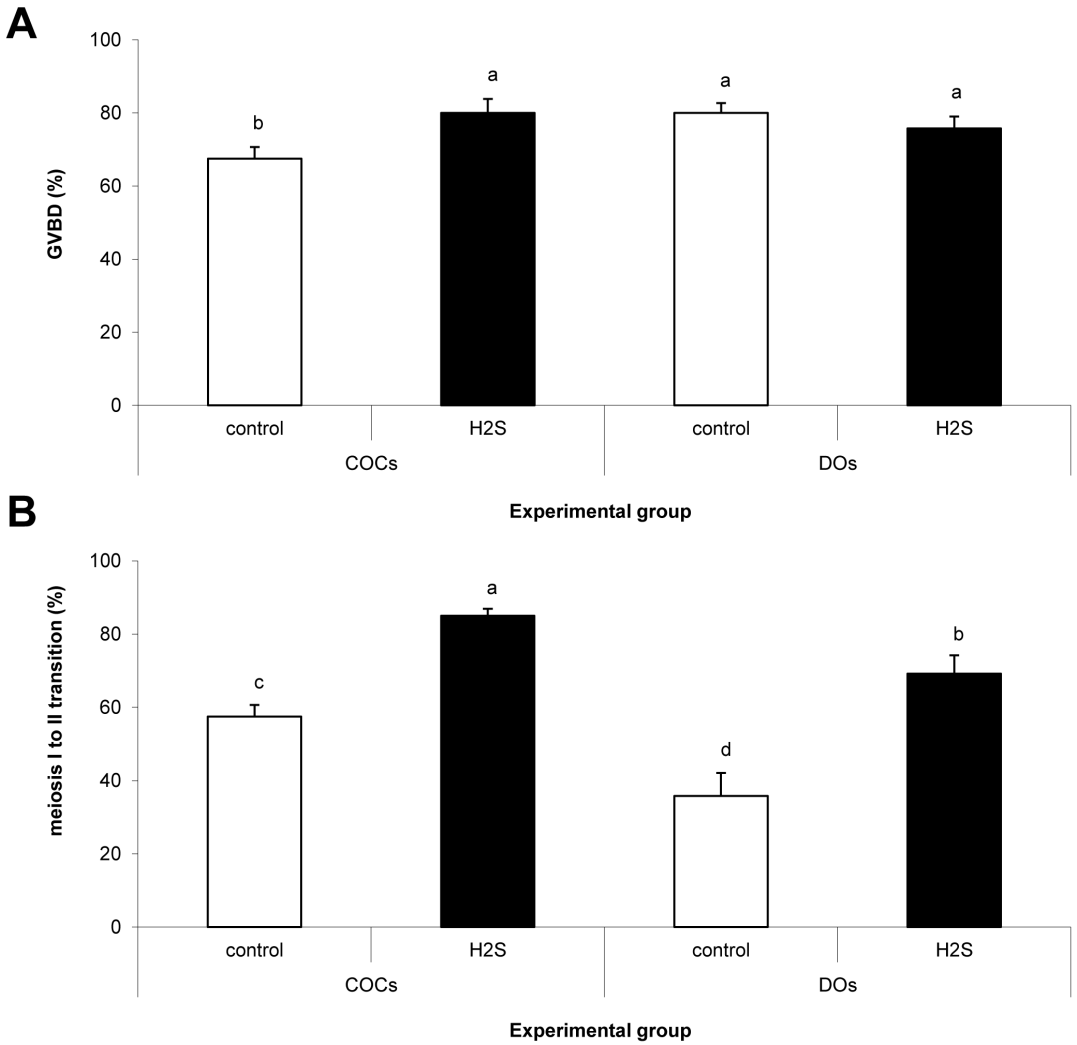

Figure 4. Effect of Na_2S on meiosis resumption and transition to meiosis II in DOs. Proportion of GVBD (A) and meiosis I to II transition (B) during *in vitro* cultivation after 20 and 30 h *in vitro* cultivation, respectively. H_2S: 300 μM Na_2S. [a,b,c]Statistically significant differences among experimental groups ($P<0.05$).
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in vitro maturation, significant acceleration of MPF occurred after 22 h cultivation.

H_2S Donor Can Substitute for the Absence of Cumulus Cells

Denuded oocytes (DOs) were cultured with the H_2S donor to evaluate cumulus cells' role during accelerated meiotic maturation. The aim of the experiment was to evaluate the GVBD and meiosis I to II transition of oocytes cultivated with 300 μM Na_2S for 20 and 30 hs, respectively. No effect of Na_2S on GVBD rates of DOs after 20 hs was observed. It should also be noted that in comparison to the control, more H_2S-treated DOs reached nuclear stages of meiosis II after 30 hs (69.2 vs. 35.8% for H_2S donor and control of DOs, respectively), see Figure 4. In addition, more DOs cultured with the H_2S donor reached metaphase II (30.0%) in comparison with the control DOs and COCs (16.7 and 6.7%, respectively) and even COCs cultured with the H_2S donor (15.8%). Further data are available in Table S3a and S3b.

H_2S Donor Influences Cumulus Expansion with Presence of Oocytes

The aim of the experiment was to measure cumulus expansion by hyaluronic acid (HA) content in COCs and OOXs. The total HA production was assessed by HA content released into the culture medium and by retained HA in cell lysate. The total and retained HA was measured in COCs after 48 h *in vitro* cultivation and during maturation after 12, 24, 36 and 48 hs. The results are compared to control COCs after 48 h cultivation. It was observed that H_2S donor, Na_2S, inhibited total HA production after 48 hs by 21.9–34.6%. No dose-dependent manner was observed, differences are statistically significant (Figure 5A). For further experiments, a concentration of 300 μM Na_2S was used.

HA production during *in vitro* cultivation of COCs is low after 12 hs of cultivation and it increased after 24 hs without significant differences between the control and H_2S groups. The H_2S donor significantly inhibited total HA production after 36 and 48 h cultivation by 13.0 and 29.0%, respectively (Figure 5B).

To evaluate the influence of oocyte presence on HA production and cumulus expansion, oocytectomised complexes (OOXs) were

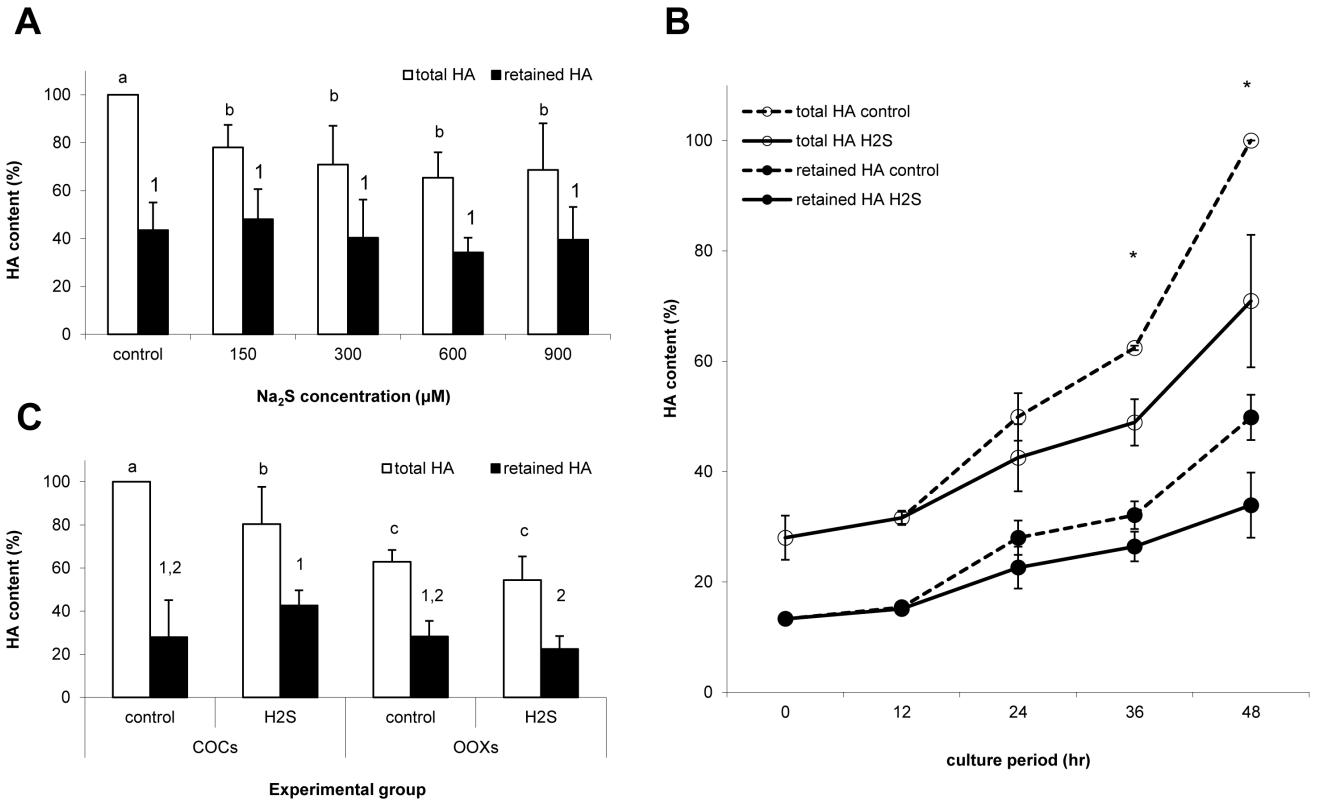

Figure 5. Effect of Na_2S on HA content in expanded cumulus. (A) Total and retained HA content in COCs cultivated with 150–900 μM Na_2S for 48 hs, total HA is related to the control group. (B) Total and retained HA content in COCs during *in vitro* cultivation with 300 μM Na_2S over 12 h time scale, total HA is related to the control group after 48 h cultivation. (C) Total and retained HA content in COCs and OOXs cultivated with or without H_2S donor, total HA is related to the control group of COCs. H_2S: 300 μM Na_2S. [a,b,c]Statistically significant differences among experimental groups in total HA, [1,2]statistically significant differences among experimental groups in retained HA, *statistically significant differences in total HA between control and H_2S groups ($P<0.05$).
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cultivated with the H_2S donor for 48 hs. It was found that oocytectomisation reduced total HA in OOXs cultivated in a pure medium by 37.0%. HA production by OOXs cultivated with H_2S donor decreased with no statistical significance in comparison with the above-mentioned OOXs. The data are shown in Figure 5C.

H_2S Donor Increases Activation Rate but It Has No Effect on Parthenogenetic Development

The influence of the H_2S donor on developmental competence acquisition during *in vitro* oocyte maturation was examined. The oocytes were matured with 300 μM Na_2S and in pure medium for 44 and 46 hs, respectively, when 100% of oocytes in both group were matured (see Table S2). The H_2S donor in maturation medium significantly increased the activation rate (91.7 vs. 75.8% for H_2S donor and control, respectively). The cleavage rate, morula and blastocyst formation were not influenced (Table 1).

Discussion

In this study, we observed the relevant impact of the exogenously added H_2S donor on porcine oocyte maturation. Originally, H_2S was described as a toxic gas [38]. However, H_2S is also endogenously generated in many types of mammalian cells, where it acts as a signal molecule, known as a gasotransmitter [2]. The concentrations of H_2S donor we used are comparable to

Table 1. Effect of Na_2S on partenogenetic development of porcine oocytes.

	Activation rate (24 hs)	n	Cleavage rate (2days)	Stage of early embryonic development (7 days)		n
				Morula	Blastocyst	
control	75.8±3.2	120	63.3±7.2	26.7±7.2	23.3±2.7	120
H_2S	91.7±3.3*	120	70.8±5.0	30.8±1.7	25.0±4.3	120

Oocytes were matured with or without Na_2S and partenogenetically activated using calcium ionophore. Pronucleus formation after 24 h zygote culture, cleavage rate after 2 days and blastocyst achievement after 7 days presumptive embryos culture were evaluated (%±SE).
H_2S: 300 μM Na_2S during oocyte maturation.
*Statistically significant differences between control and H_2S group – in column ($P<0.05$).
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physiological values in tissues [2,3] and we could assume that the observed effects of H_2S donor exogenously added were not a result of its toxicity but rather relied on the physiological effect of H_2S as a gasotransmitter. To the best of our knowledge, this study is the first one to describe the influence of the H_2S donor on meiotic maturation of oocytes.

Significant acceleration of oocyte maturation during *in vitro* cultivation of porcine cumulus-oocyte complexes (COCs) with the H_2S donor was observed. In agreement with a former study [34], meiotic maturation of oocytes was accelerated by an earlier increase of MPF and MAPK regulating oocyte maturation. The mechanisms underlying this precocious activation of MPF/MAPK induced by H_2S remain to be determined. It is known that H_2S can influence the activity of various factors including kinases by their direct sulfhydration [39], but no direct effect of H_2S on MPF and MAPK activities has been yet reported. In addition to possible direct regulation, H_2S may act indirectly on kinase activity by modifying other molecules, such as ion channels [40], and/or through regulation of up-stream kinases [30,31]. Thus, the sulfhydration of these proteins may tune and control the oocyte maturation processes. In somatic cells, H_2S-stimulation of signal pathways of cAMP/PKA [32] and PI3K/Akt [31] was observed. The important contribution these signal pathways make to kinase activity control during mammalian oocyte maturation is known [10,41]. The experiments undertaken demonstrate that the H_2S donor does not suppress acquisition of oocyte developmental competence during their *in vitro* maturation.

In our experiments, the action of the H_2S donor on oocyte maturation in porcine COCs poses the question of whether the H_2S donor effect is the result of direct function in oocytes, or whether the action of exogenous H_2S is transduced by cumulus cells. Our results suggest that the H_2S donor acts directly on the oocyte. Indeed, accelerated maturation by the H_2S donor was observed in denuded oocytes (DOs) cultivated after removal of cumulus cells. The acceleration of meiotic maturation in H_2S donor treated DOs was even more marked than in treated COCs. An explanation for this phenomenon could be in exogenous H_2S retention in cumulus cells and/or in the extracellular matrix produced by these cells. This results in a smaller quantity of H_2S being available for the oocytes. In addition, the H_2S donor may cause processes inhibiting oocyte maturation in cumulus cells [42]. Accordingly, the immediate H_2S donor influence induces faster meiotic maturation of DOs.

The influence of the H_2S donor on cumulus cells was demonstrated by our subsequent experiments, in which we measured the level of hyaluronic acid (HA) in the extracellular matrix of cumulus cells as a marker of cumulus expansion. We showed inhibition of HA production in COCs cultivated with the H_2S donor. The effect of the H_2S donor on HA production was observed in all the concentrations of the H_2S donor used after 48h *in vitro* cultivation. The H_2S donor significantly influenced HA production in the second moiety of COC cultivation. A previous study had illustrated the decrease in activity of factors stimulating cumulus expansion, such as Cumulus Expansion Enabling Factor (CEEF), after metaphase I attainment [43]. We can presume that the role of the H_2S donor may be in the deepening of CEEF decrease. The mechanism of H_2S effect on cumulus expansion is as yet unclear. One possibility could be the influence of the above-mentioned cAMP/PKA signal pathway [32], which regulates cumulus expansion [44].

Cumulus expansion is extensively regulated by substances with oocyte origin [43,44]. For this reason, we evaluated the influence of oocyte presence on cumulus expansion during cultivation with the H_2S donor. We measured HA production in oocytectomied complexes (OOXs) where the oocyte had been removed. Our observation of decreased HA production after oocytectomy is in line with the previous study [45], where a decline of HA-synthase 2 expression in cumulus cells was shown. Whereas we demonstrated the inhibition of HA production in intact COCs cultivated with the H_2S donor, no effect was observed in OOXs. It is known that production of CEEF by porcine cumulus cells is sufficient for cumulus expansion [35]. However, our experiments showed that inhibition of cumulus expansion by the H_2S donor is mediated by the oocyte. Target systems in oocytes for H_2S, regulating HA production in this way, remain unknown. Presumably, possible target molecules for exogenous H_2S might be some members of the Transforming Growth Factor β superfamily which can be regulated by H_2S [46] and subsequently influence HA-synthase 2 activity in cumulus cells [47].

The results of our study demonstrate that the H_2S donor can participate in the regulation of oocyte maturation and cumulus expansion without the interference of developmental competence acquired during *in vitro* maturation. Further experiments are necessary for a full explanation of the role of H_2S as a signal molecule and the mechanism of its effect during oocyte maturation, cumulus expansion and early embryogenesis.

Supporting Information

Figure S1 Effect of Na_2S on kinase activity during oocyte cultivation. Representative autoradiograms and signal quantifications of phosphorylated histone H1 (A) and MBP (B) reflecting MPF and MAPK activity, respectively. Kinase activity was measured in oocytes cultivated with or without Na_2S in 6 hr time scale. The kinase activity was related to oocytes cultivated for 24 hrs. C: control; H_2S: 300 μM Na_2S. *Statistically significant differences between control and H_2S group ($P<0.05$).
(TIF)

Table S1 Effect of different Na_2S concentrations on oocyte maturation after 20 hr (S1a) cultivation and 30 hr cultivation (S1b).
(DOC)

Table S2 Effect of 300 μM Na_2S on oocyte maturation.
(DOC)

Table S3 Effect of Na_2S on maturation of DOs after 20 hr (S3a) cultivation and 30 hr cultivation (S3b).
(DOC)

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Author Contributions

Conceived and designed the experiments: JN JP JFB MS. Performed the experiments: JN AG TK TK VKC MD AV IW LK TZ. Analyzed the data: JN JP FJ. Wrote the paper: JN JP FJ.

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5.2 Vliv suplementace kultivačního média donorem sulfanu během prodloužené kultivace prasečích oocytů

Sulfan je endogenně produkován během stárnutí prasečích oocytů. Endogenní produkce sulfanu klesá po prvních 24 hodinách prodloužené kultivace o 29 %. Stanovená hypotéza, že suplementace kultivačního média donorem sulfanu může zvýšit kvalitu oocytů vystavených prodloužené kultivaci, byla ověřena prostřednictvím Na_2S, který byl přidáván do kultivačního média pro stárnoucí oocyty. Suplementace kultivačního média donorem sulfanu potlačila projevy stárnutí oocytů vystavených prodloužené kultivaci a zlepšila jejich časný embryonální vývoj.

Vliv donoru sulfanu a inhibitorů sulfan uvolňujících enzymů na stárnutí oocytů byl hodnocen morfologickým hodnocením stárnoucích oocytů. Během prodloužené kultivace docházelo ke snížení kvality stárnoucích oocytů, po 48 hodinách stárnutí bylo v kontrolní skupině 18,3 % oocytů fragmentovaných a po 72 hodinách stárnutí byl podíl fragmentovaných oocytů 25,9 %. Suplementace média Na_2S (0,3 mM) zcela potlačila fragmentaci oocytů po 48 a 72 hodinách prodloužené kultivace.

Inhibice aktivity sulfan uvolňujících enzymů naopak způsobila výskyt fragmentovaných (6,6 – 13,3 %) a spontánně partenogeneticky aktivovaných (12,5 – 21,7 %) oocytů již po 24 hodinách prodloužené kultivace, kdy se v kontrolní skupině se nacházely pouze intaktní oocyty. Inhibice dvou anebo všech tří sulfan uvolňujících enzymů vedla k výraznějšímu výskytu projevů stárnutí

Kvalitu oocytů vystavených účinkům inhibitorů endogenní produkce sulfanu lze zlepšit exogenně dodaným donorem sulfanu. Po 72 hodinách prodloužené kultivace suplementace média Na_2S (0,3 mM) zcela potlačila fragmentaci oocytů ošetřených kombinací inhibitorů CBS a CSE a kombinací inhibitorů CSE a 3-MPST a částečně potlačila i fragmentaci oocytů ošetřených kombinací inhibitorů CBS a 3-MPST (z 24,2 % na 10 %) a kombinací inhibitorů všech tří sulfan uvolňujících enzymů (z 65,8 % na 20,5 %).

Přestože suplementace média Na_2S a inhibitory sulfan uvolňujících enzymů ovlivnila výskyt morfologických projevů stárnutí oocytů, Na_2S a inhibitory sulfan uvolňujících enzymů neměly vliv na aktivitu MPF a MAPK během prodloužené kultivace oocytů.

Oocyty vystavené 24 hodinám prodloužené kultivace s donorem sulfanu (0,15 mM) se ve zvýšené míře rýhovaly 48 hodin po partenogenetické aktivaci (54,2 % vs. 41,7 %) a ve zvýšené míře dosahovaly stádií moruly (20,8 % vs. 8,3 %) a blastocysty (15,8 % vs. 1,7 %)

sedm dní po partenogenetické aktivaci ve srovnání s oocyty vystavenými 24 hodinám prodloužené kultivace v médiu bez donoru sulfanu.

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PLOS ONE

RESEARCH ARTICLE

Hydrogen Sulfide Donor Protects Porcine Oocytes against Aging and Improves the Developmental Potential of Aged Porcine Oocytes

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Abstract

Porcine oocytes that have matured in in vitro conditions undergo the process of aging during prolonged cultivation, which is manifested by spontaneous parthenogenetic activation, lysis or fragmentation of aged oocytes. This study focused on the role of hydrogen sulfide (H_2S) in the process of porcine oocyte aging. H_2S is a gaseous signaling molecule and is produced endogenously by the enzymes cystathionine-β-synthase (CBS), cystathionine-γ-lyase (CSE) and 3-mercaptopyruvate sulfurtransferase (MPST). We demonstrated that H_2S-producing enzymes are active in porcine oocytes and that a statistically significant decline in endogenous H_2S production occurs during the first day of aging. Inhibition of these enzymes accelerates signs of aging in oocytes and significantly increases the ratio of fragmented oocytes. The presence of exogenous H_2S from a donor ($Na_2S.9H_2O$) significantly suppressed the manifestations of aging, reversed the effects of inhibitors and resulted in the complete suppression of oocyte fragmentation. Cultivation of aging oocytes in the presence of H_2S donor positively affected their subsequent embryonic development following parthenogenetic activation. Although no unambiguous effects of exogenous H_2S on MPF and MAPK activities were detected and the intracellular mechanism underlying H_2S activity remains unclear, our study clearly demonstrates the role of H_2S in the regulation of porcine oocyte aging.

Introduction

Porcine oocytes, similarly to the majority of mammal oocytes, can be fertilized in the MII stage of meiotic maturation. If oocytes are not fertilized shortly after the completion of meiotic

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maturation, then they undergo a number complex undesirable changes called aging [1,2]. Their quality and capacity to undergo proper further embryonic development after fertilization rapidly decrease [3].

Oocytes undergo functional and morphological changes during aging. Among other contributing factors, oocyte aging is partly due to changes in M-phase promoting factor (MPF) and mitogen-activated protein kinase (MAPK) activity, which are necessary to maintain meiotic arrest in metaphase II [4,5]. Diminution of MAPK activity and MPF inactivation leads to one of the main manifestations of aging: spontaneous parthenogenetic activation. Aged oocytes may also undergo fragmentation (apoptosis) induced by a high level of MAPK activity, or lysis [6–9].

Hydrogen sulfide (H_2S) is one of the upstream factors that control MAPK activity [10]. H_2S, a gaseous mediator, is produced in cells from the amino acid L-cysteine by three enzymes: cystathionine-β-synthase (CBS), cystathionine-γ-lyase (CSE) and 3-mercaptopyruvate sulfurtransferase (MPST). The expression and activity of these enzymes vary in different tissues [11,12]. The expression of these enzymes and endogenous H_2S production from tens to hundreds of micromoles have been described in the central nervous and the respiratory system [13–15]. H_2S is also involved in the regulation of reproduction.

CBS and CSE expression, but not MPST, have been reported in mouse, rat and human reproductive systems [16,17]. CBS knockout mice have reduced quantities of growing follicles and irregular, shorter estrus cycles [18,19]. Liang et al. [20] demonstrated the presence of CBS in follicular and granulose cells but not in oocyte alone. However, decreased CBS expression in granulose cells has been associated with the inhibition of meiotic maturation in mouse oocytes [21].

The requirement for H_2S production by cumulus cells for proper porcine oocyte meiotic maturation has been described by Nevoral et al. [22]. H_2S, by regulating ion channels and kinase activities, participates in the regulation of apoptosis in somatic cells. Its effect can be pro-apoptotic or anti-apoptotic depending on the situation and type of cell [23–26].

We hypothesized that endogenous production of H_2S is involved in the regulation of porcine oocyte aging and that oocyte aging can be affected by exogenous H_2S. The aim of this study was to detect the endogenous production of H_2S in porcine oocytes and to assess its involvement in oocyte aging. Additional aims of the study were to determine whether H_2S participates in the regulation of MPF and MAPK activities, including whether exogenous H_2S can suppress the manifestations of aging and improve the quality of aged oocytes in relation to consecutive embryonic development.

Materials and Methods

Collection and Cultivation of Oocytes

Porcine ovaries were obtained from a local slaughterhouses in Cesky Brod and Pilsen from gilts (Large White × Landrace, slaughter weight 110 kg, 6 months old) during an unknown stage of the oestrous cycle and were transported to the laboratory in a saline solution (0.9% natrium chloride) at 39°C. Oocytes were obtained through the aspiration of follicles (2 to 5 mm in diameter) with a 20-gauge needle. Only oocytes with compact cumuli were chosen for experiments. Oocytes were washed three times in the culture medium before cultivation. Oocytes were cultivated in a modified M199 medium (GibcoBRL, Life Technologies, Carlsbad, USA) containing sodium bicarbonate (32.5 mM; Sigma-Aldrich, USA), calcium L-lactate (2.75 mM; Sigma-Aldrich, USA), sodium pyruvate (0.25 mg/ml; Sigma-Aldrich, USA), gentamicin (0.025 mg/ml; Sigma-Aldrich, USA), HEPES (6.3 mM; Sigma-Aldrich, USA), 10% (v/v) foetal

calf serum (Gibco BRL, Life Technologies, Germany) and 13.5 IU eCG: 6.6 IU hCG/ml (P.G. 600, Intervet, Boxmeer, Netherlands).

The oocytes were then cultured in 3.5 cm diameter Petri dishes (Nunc, Roskilde, Denmark) containing 3 ml of culture medium at 39°C. The Petri dishes were placed into incubator in a mixture of 5% CO_2 in air for 48 hours, until oocytes reached the metaphase of the second meiotic division (MII). Subsequently, oocytes were denuded of cumular cells by repeated pippeting through a thin glass capillary and then cultivated according to specific experimental procedures.

In Vitro Cultivation of Aging Oocytes with H_2S Donor and Inhibitors of H_2S-producing Enzymes

After denuding the oocytes in metaphase II, they were exposed to prolonged cultivation (aging) in a modified medium M199 (GibcoBRL, Life Technologies, Carlsbad, USA) without P. G. 600. Once again oocytes were cultivated in 3.5 cm diameter Petri dishes (Nunc, Roskilde, Denmark), containing 3 ml of culture medium at 39°C. The Petri dishes were placed into an incubator in a mixture of 5% CO_2 in air for 24, 48 and 72 hours. The hydrogen sulfide donor, $Na_2S.9H_2O$ (Sigma-Aldrich, USA), in its effective concentrations was used. Effective concentrations of this H_2S donor were selected after the previous testing. To monitor the effect of elevated levels of H_2S on porcine oocytes aging, the concentration 300 μM of $Na_2S.9H_2O$ was selected because the fragmentation of the porcine oocytes cultivated in medium supplemented with such concentration of $Na_2S.9H_2O$ after 72 hours failed to occur as well as the highest number of oocytes were still in metaphase II (see Supporting Information, S1 Table).

Specific inhibitors were used to inhibit the enzymes activity. We used 1 mM oxamic acid (OA; Sigma-Aldrich, USA) as a CBS inhibitor, 1 mM beta-kyano-L-alanine (KA; Sigma-Aldrich, USA) as a CSE inhibitor and 5mM alpha-ketoglutaric acid disodium salt dihydrate (KGA; Sigma-Aldrich, USA) as a MPST inhibitor. These inhibitors were used alone or in various combinations (1 mM OA + 1 mM KA; 1 mM OA + 1 mM KGA; 1 mM KA + 5 mM KGA; 1 mM OA + 1 mM KA + 5 mM KGA). Each concentration of the inhibitors was selected after the previous testing (data not shown). Experiments were repeated three times and a minimum of 120 oocytes were evaluated in each experiment.

Parthenogenetic Activation of Oocytes

Oocytes were activated using calcium ionophore A23187 (25 μM, 5 min; Sigma-Aldrich, USA) combined with a 6-dimethyl aminopurine - 6-DMAP (2 mM, 2 h; Sigma-Aldrich, USA) treatment [27]. In experimental groups, porcine oocytes were exposed to prolonged cultivation (aging) for 24 hours in medium M199 supplemented with hydrogen sulfide donor, $Na_2S.9H_2O$ in concentrations 150 μM, or 300 μM. These concentrations were selected after previous testing, because in the group of porcine oocytes aged 72 hours in medium supplemented with 150 μM, or 300 μM of $Na_2S.9H_2O$, fragmented oocytes failed to occur (see Supporting Information, S1 Table).

Parthenogenetically activated oocytes were subsequently cultivated 24 hours. Oocytes with formed pronuclei were considered parthenogenetically activated. Experiments were repeated three times and a minimum of 120 oocytes were evaluated in each experiment.

In Vitro Cultivation of Parthenogenetic Embryos

Parthenogenetically activated oocytes were cultured in a NCSU 23 medium [28] in 4-cell Petri dishes (Nunc, Roskilde, Denmark) containing 1 ml of culture medium at 39°C. The Petri dishes were placed into incubator in a mixture of 5% CO_2 in air. The ratio of embryo cleavage was

determined after 48 hours of culture. The ratio of embryos in the morula and blastocyst stage was determined after 168 hours (7 days) of culture. Experiments were repeated three times and a minimum of 120 oocytes were evaluated in each experiment.

Evaluation of Oocytes and Parthenogenetic Embryos

At the completion of the cultivation period, the oocytes and embryos were fixed in an ethanol and acetic acid (3:1, v/v) for 48 hours. Subsequently, fixed oocytes and embryos were stained with 1.0% (w/v) orcein in 50% aqueous-acetic acid. Oocytes and embryos were then examined under a phase-contrast microscope. Aged oocytes were classified into four groups: intact oocytes (oocytes at metaphase II, anaphase II or telophase II); activated oocytes (oocytes with pronuclei or embryos); fragmented oocytes (oocytes were designated as fragmented when fragmented "vesicles" were observed under the *zona pellucida*), and lysed oocytes (rupture of the cytoplasmic membrane and loss of the integrity of the oocyte were the criteria for lysis) (see Fig. 1; [1]).

Measurement of Hydrogen Sulfide Production

H_2S production in porcine oocytes was verified through spectrophotometric analysis of S^{2-} ions in accordance with Stipanuk and Beck [29], modified by Abe and Kimura [30] and Pan et al., [31], modified for porcine oocytes. Samples from oocytes at metaphase II, as well as samples from oocytes exposed to aging for 24 hours were prepared for this experiment. Each sample comprised 100 oocytes.

Initially, oocytes were mechanically disrupted in the presence of the following reaction mixture (500 μl) under gaseous nitrogen N_2 and low temperature (4°C) conditions: pyridoxal 5-phosphate (0.2 M, 5 μl, Sigma-Aldrich, USA) L-cystein (10 mM, 50 μl, Sigma-Aldrich, USA) and deionized water (445 μl).

Subsequently, zinc acetate (1%, 250 μl, Sigma-Aldrich, USA) was added into the reaction mixture. The reaction of the enzymes was initiated by increasing the temperature of the tube from 4°C to 37°C. After the culture (60 min), 50% trichloroacetic acid (250 μl, Sigma-Aldrich, USA) was added to the reaction mixture to stop the reaction. Subsequently, the reaction mixture was incubated for the next 60 minutes at 37°C. Afterwards, the N,N dimethyl-p-phenylenediamine sulphate (20 mM in 7.2 M HCl, 133 μL, Sigma-Aldrich, USA) and $FeCl_3$ (30 mM in 1.2 M HCl, 133 μL, Sigma-Aldrich, USA) were added. The sample absorbance was measured by a spectrophotometer at 670 nm. The results of these measurements were presented as a ratio relative to the group of oocytes at metaphase II, which was taken as a value of 100%.

Histone H1 and Myelin Basic Protein Double Assay

Histon H1 kinase assay and MBP (Myelin basic protein) were carried out to determine MPF and MAPK activity by measuring their capacity to phosphorylate their substrates (histon H1 and MBP—Myelin basic protein) [22]. Histon H1 kinase and MBP assays were determined in oocytes at metaphase II, oocytes aged for 12 and 24 hours in modified M199 medium, oocytes aged for 12 and 24 hours in modified M199 medium supplemented with 300 μM of $Na_2S.9H_2O$ and oocytes aged for 12 and 24 hours in modified M199 medium supplemented with 1 mM Oxamic acid + 1 mM beta-kyano-L-alanine + 5 mM alpha-ketoglutaric acid disodium salt dihydrate. Samples were immediately frozen to −80°C. The data obtained were analysed and evaluated by MultiGauge 2.0 software. The results were presented as the ratio relative to the activity of group of oocytes at metaphase II, which was taken as a value 1.

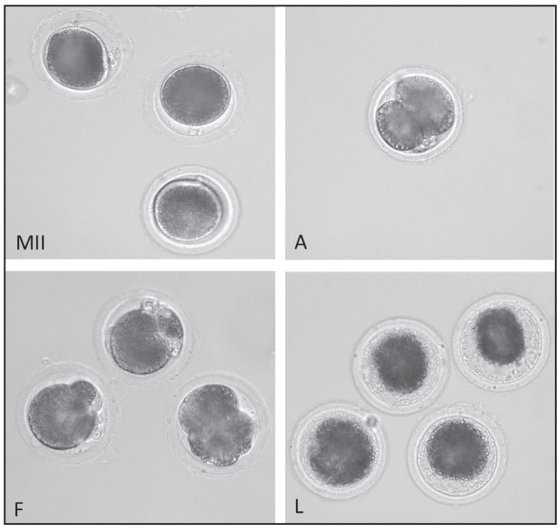

Figure 1. Effect of aging on morphology of porcine oocyte. MII—intact oocyte at metaphase II, A—activated oocyte, F—fragmented oocyte, L—lysed oocyte.

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Statistical analysis

Data from all the experiments was subjected to statistical analysis. The SAS 9.0 software (SAS Institute Inc., USA) was used for statistical analysis and data. Significant differences between groups were determined using the ANOVA test. An a P value of less than 0.05 was considered significant.

Ethics Statements

All animal work was conducted according to Act No 246/1992 Coll., on the protection of animals against cruelty under supervision of Central Commission for Animal Welfare, approval ID 018/2010. The approval of Institutional Animal Care and Use Commitee (IACUC) was not required as the experimens were performed under in vitro conditions.

Results

Endogenous Production of H_2S Decreases During the First 24 hours of Aging

In this experiment, we focused on evaluating endogenous H_2S production during the porcine oocyte aging process. We demonstrated that H_2S is enzymatically produced in porcine oocytes through H_2S producing enzyme activity. The highest level of H_2S production occurred in mature oocytes that were not subjected to aging. Endogenous production of H_2S significantly decreased by 29% as early as at 24 hours of oocyte aging compared with mature oocytes in metaphase II that were not subjected to aging (see Fig. 2).

Exogenous H_2S Protects Oocytes Against Aging

In the next experiment, we attempted to influence the signs of aging by compensating for the decrease in H_2S production with exogenous H_2S by cultivating the oocytes in a medium containing an H_2S donor.

A gradual, significant increase in the proportion of spontaneously parthenogenetically activated and fragmented oocytes was monitored during porcine oocyte aging *in vitro*. Oocytes

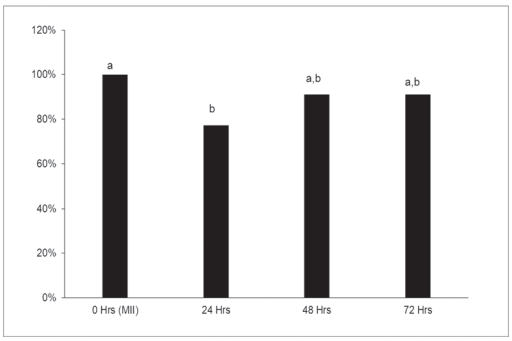

Figure 2. Determination of endogenous H_2S production during porcine oocyte aging. Oocytes were cultivated to metaphase II (MII). Hydrogen sulfide production was carried out using the spectrophotometric method. MII oocytes, as well as oocytes exposed to prolonged cultivation for 24, 48 and 72 hours, were examined. The results of the measurement are presented as a ratio relative to the MII oocyte group. *[a, b] Statistically significant differences in spontaneous hydrogen sulfide production are indicated by different superscripts ($P<0.05$). Each experiment was repeated four times. The total number of oocytes in each sample was 100.*

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with pronuclei or embryos were classified as parthenogenetically activated and oocytes with fragmented "vesicles" under the *zona pellucida* were classified as fragmented. Oocyte at MII, AII and TII were considered intact (see Fig. 1). After 48 hours of aging, 37.2 ± 2.5% of the oocytes were parthenogenetically activated and 18.3 ± 2.9% were fragmented in the control group; after 72 hours of aging, 47.5 ± 2.5% of the oocytes were parthenogenetically activated and 25.9 ± 2.9% were fragmented.

The presence of H_2S donor (300 μM $Na_2S.9H_2O$) in the culture medium completely suppressed the fragmentation of aging oocytes. After 48 hours of aging, only intact oocytes (76.7 ± 3.8%) and parthenogenetically activated oocytes (23.3 ± 3.8%) were observed in the experimental group. Fragmented oocytes were not detected in the experimental group, even after 72 hours of aging (55.0 ± 5.0% intact oocytes and 45.0 ± 5.0% parthenogenetically activated oocytes) (Fig. 3A, for detailed results see Supporting Information S2, S3, S4 Tables).

Inhibition of Endogenous H_2S Production Accelerates the Oocyte Aging Process

In this experiment, we focused on monitoring the effects of inhibitors of individual enzymes (CBS, CSE, MPST) that are responsible for the endogenous production of H_2S. Inhibition of these enzymes led to earlier onset of signs of aging in porcine oocytes.

Oocytes that were aged in medium containing inhibitors, in contrast to the control group, were already parthenogenetically activated or fragmented during the first 24 hours of aging. In contrast, 100% of the oocytes in the control group were in metaphase II during the first 24 hours of aging (Fig. 3A), and among oocytes cultured in the presence of alpha-ketoglutaric acid disodium salt dihydrate (inhibitor of MPST), 13.3 ± 2.9% of the oocytes were parthenogenetically activated and 12.5 ± 0.0% were fragmented. In the group of oocytes cultured in the presence of beta-kyano-L-alanine (inhibitor of CSE) and oxamic acid (inhibitor of CBS), 7.5 ± 2.5% and 6.6 ± 1.4% of the oocytes were parthenogenetically activated and 20.0 ± 2.5% and 21.7 ± 3.8% were fragmented, respectively.

The effect of inhibitors decreased gradually after 48 and 72 hours of oocyte aging. After 72 hours of aging, we observed a significant difference in comparison to the control group only in the group of oocytes treated with MPST inhibitor (47.5 ± 2.5% vs 22.0 ± 3.5%

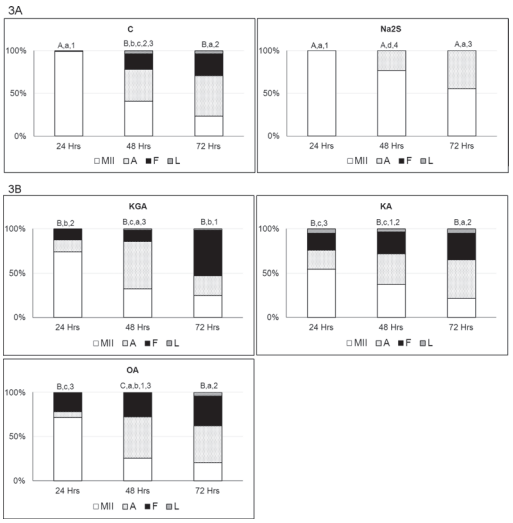

Figure 3. Effects of an elevated H_2S level and inhibition of H_2S producing enzymes during oocyte aging. Oocytes were cultivated to metaphase II and then exposed to prolonged cultivation in a modified M199 medium for 24, 48 and 72 hours in the presence of a H_2S donor or H_2S producing enzymes inhibitors. **3A**. Na_2S ($Na_2S.9H_2O$; 300 μM) was used as the H_2S donor. **3B**. Oxamic acid (1mM, OA) was used as a CBS inhibitor, beta-kyano-L-alanine (1mM, KA) was used as a CSE inhibitor and alpha-ketoglutaric acid disodium salt dihydrate (5mM, KGA) was used as a MPST inhibitor. *C- control; Na_2S (300 μM, $Na_2S.9H_2O$); KGA—alpha-ketoglutaric acid disodium salt dihydrate (5 mM); KA – beta-kyano-L-alanine (1mM); OA—oxamic acid (1 mM); MII—intact oocytes (oocytes at metaphase II, anaphase II or telophase II), A—activated oocytes (oocytes with pronuclei or embryos), F—fragmented oocytes, L—lysed oocytes. Different letters and numbers indicate significant differences between different treatments and hours of aging ($P<0.05$). A,B,C – statistically significant differences in portion of MII stage oocytes between individual treatments. a,b,c,d – statistically significant differences in portion of activated oocytes between individual treatments. 1,2,3 – statistically significant differences in portion of fragmented oocytes between individual treatments*.

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parthenogenetically activated oocytes and 25.9 ± 2.9% vs 51.3 ± 4.3% fragmented oocytes) (Fig. 3B). For detailed results, refer to Supporting Information S2, S3, S4 Tables.

Inhibition of Endogenous H_2S Production Can Be Reversed by Exogenous H_2S

The specific effect of inhibitors was verified by the reversion of this inhibitory effect using the H_2S donor $Na_2S.9H_2O$ at a concentration of 300 μM.

The effect of CBS and CSE inhibitors could be completely reversed by the addition of H_2S donor. The H_2S donor reversed the effect of the MPST inhibitor but only in the presence of fragmented oocytes (Fig. 4). For detailed results, refer to Supporting information S5 Table.

Effect of Double or Triple Inhibition of H_2S-Producing Enzymes Can Be Reversed by Exogenous H_2S

Subsequently, we focused on the possibility of supplementing endogenous H_2S production with an exogenous donor to determine the effect on oocyte aging. We studied the effect of H_2S donor on oocyte aging after inhibiting two or all three H_2S-producing enzymes.

Although the effects of a combination of inhibitors differed from one another, in all of the experimental groups, the presence of exogenous H_2S significantly suppressed the fragmentation of aging oocytes. Fragmentation was completely suppressed by the application of exogenous H_2S in the group of oocytes that were aged in the presence of two inhibitors: inhibitors of CBS and CSE (0.0% vs 25.8 ± 3.8%; 72 hours of aging) and inhibitors of CSE and MPST (0.0%

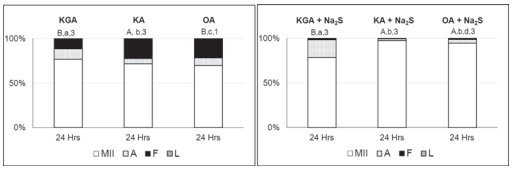

Figure 4. Reversion of the effects of CBS, CSE and MPST inhibitors using a H_2S donor. Oocytes were cultivated to metaphase II and then exposed to prolonged cultivation (24 hours) in a modified M199 medium supplemented with a H_2S donor ($Na_2S.9H_2O$; 300 μM) and the following individual inhibitors: oxamic acid (1mM, OA), beta-kyano-L-alanine (1mM, KA), and alpha-ketoglutaric acid disodium salt dihydrate (5mM, KGA). *Na_2S (300 μM, $Na_2S.9H_2O$); KGA—alpha-ketoglutaric acid disodium salt dihydrate (5 mM); KA – beta-kyano-L-alanine (1mM); OA—oxamic acid (1 mM); MII—intact oocytes (oocytes at metaphase II, anaphase II or telophase II), A—activated oocytes (oocytes with pronuclei or embryos), F—fragmented oocytes, L—lysed oocytes; Different letters and numbers indicate significant differences between different treatments and hours of aging ($P<0.05$). A,B – statistically significant differences in portion of MII stage oocytes between individual treatments. a,b,c,d – statistically significant differences in portion of activated oocytes between individual treatments. 1,2,3 – statistically significant differences in portion of fragmented oocytes between individual treatments*.

doi:10.1371/journal.pone.0116964.g004

vs 49.2 ± 1.4%; 72 hours of aging). The effect of inhibition by CBS and MPST together was partially reversed by the application of exogenous H_2S (10.0 ± 0.0% vs 24.2 ± 3.8%; 72 hours of aging).

Simultaneous inhibition of all three H_2S-producing enzymes led to a significant detrimental effect on oocyte aging with the largest proportion of parthenogenetically activated and fragmented oocytes. Although the reversal of this inhibitory effect by the H_2S donor had a reduced efficiency in this case, it was significant (20.5 ± 4.0% vs 65.8 ± 2.9%; 72 hours of aging) (see Fig. 5; Supporting Information S6, S7, S8 Tables).

MPF and MAPK Activity is Influenced by the H_2S Level in Aging Oocytes

We monitored changes in the dynamics of key regulatory factors of meiotic maturation: MAPK and MPF. We observed a statistically significant decrease in the activity of MPF in oocytes that were cultured in the presence of H_2S donor compared to those cultured in the presence of all three inhibitors of H_2S-producing enzymes after 12 hours of aging (Fig. 6A). Conversely, MAPK activity decreased significantly after 24 hours of aging in oocytes that were cultured in the presence of inhibitors of H_2S-producing enzymes compared to those cultured in the presence of the H_2S donor (Fig. 6B).

The H_2S Donor Impairs the Efficiency of Induced Parthenogenetic Activation of Aged Porcine Oocytes

The next aim of this study was to evaluate the effect of exogenous H_2S on the activating potential of aged oocytes. We wanted to investigate whether it was possible to parthenogenetically activate porcine oocytes that had been aged in the presence of elevated levels of H_2S, with calcium ionophore and whether this treatment would have the same effect on their subsequent early embryonic development.

Following the parthenogenetic activation of oocytes that were not exposed to aging, 94.2 ± 5.2% of the oocytes were successfully activated (Table 1). Aging of oocytes for 24 hours led to a statistically significant decrease in the activation efficiency (to 79.2 ± 3.8%).

The presence of an H_2S donor did not increase the activation efficiency. Conversely, a statistically significant decrease in the portion of successfully activated oocytes was evident (to 42.5 ± 5.0) in the group of oocytes that was aged in medium supplemented with 300 μM of $Na_2S.9H_2O$.

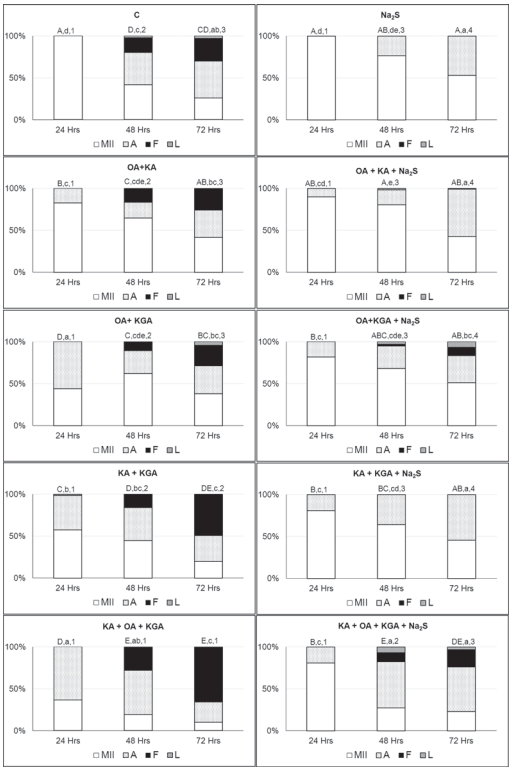

Figure 5. Effects of concurrent CBS, CSE and MPST inhibition and its reversion using a H_2S donor. Oocytes were cultivated to metaphase II and then exposed to prolonged cultivation in a modified M199 medium supplemented with a H_2S donor ($Na_2S.9H_2O$; 300 μM) and the inhibitors for 24, 48 and 72 hours. Various combinations of oxamic acid (1mM, OA) which was used as a CBS inhibitor, beta-kyano-L-alanine (1mM, KA) which was used as a CSE inhibitor and alpha-ketoglutaric acid disodium salt dihydrate (5mM, KGA) which was used as a MPST inhibitor were used in this experiment. To reverse effects of inhibitors, a H_2S donor (300 μM, $Na_2S.9H_2O$) was added to each experimental group. *C- control; Na_2S (300 μM, $Na_2S.9H_2O$); KGA—alpha-ketoglutaric acid disodium salt dihydrate (5 mM); OA—oxamic acid (1 mM); KA – beta-kyano-L-alanine (1mM); MII—intact oocytes (oocytes at metaphase II, anaphase II or telophase II), A—activated oocytes (oocytes with pronuclei or embryos), F—fragmented oocytes, L—lysed oocytes; Different letters and numbers indicate significant differences between different treatments and hours of aging ($P<0.05$). A,B,C,D – statistically significant differences in portion of MII stage oocytes between individual treatments. a,b,c,d,e – statistically significant differences in portion of activated oocytes between individual treatments. 1,2,3,4 – statistically significant differences in portion of fragmented oocytes between individual treatments*.

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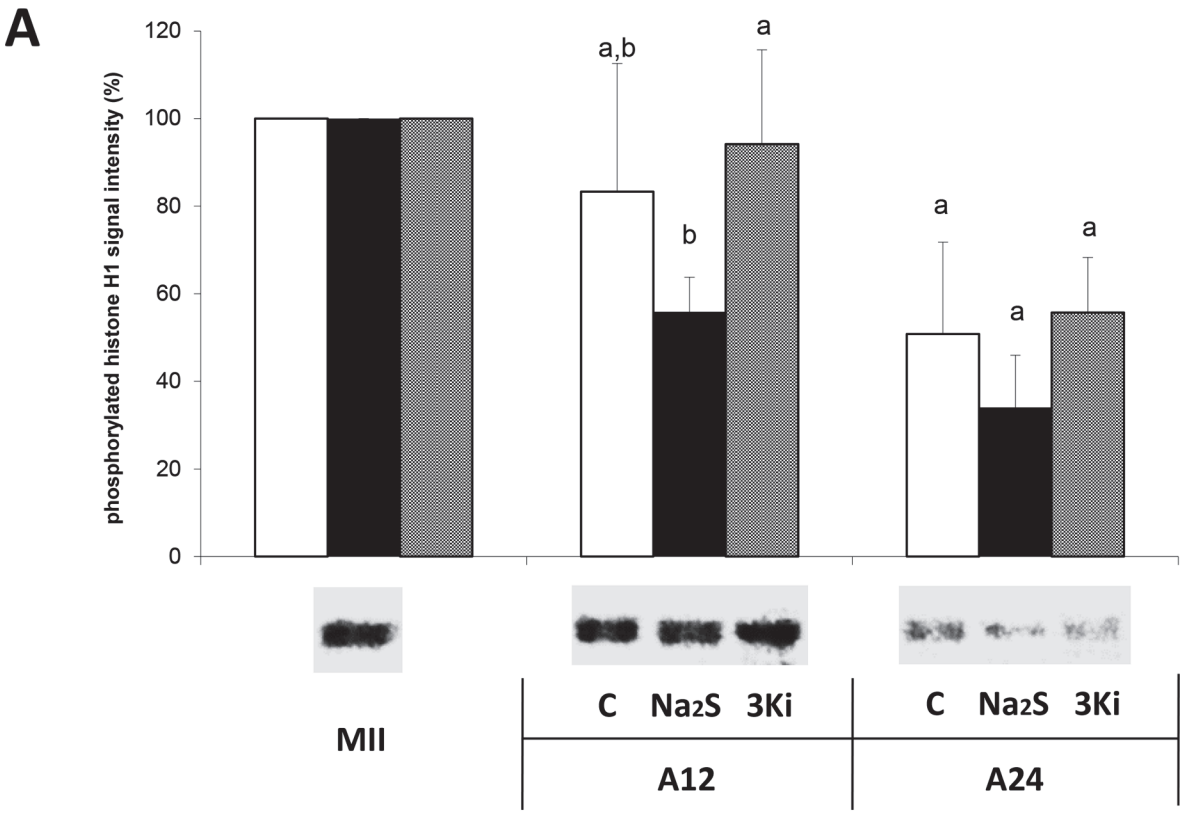
The H_2S Donor Improves Embryonic Development Following Induced Parthenogenetic Activation of Aged Porcine Oocytes

In this experiment, we focused on the possibility of influencing the developmental competence of parthenogenetically activated aged oocytes by cultivating them in the presence of H_2S donor during aging. The oocytes that were aged for 24 hours in medium supplemented with H_2S donor (150 μM) showed significantly better early embryonic development in comparison to oocytes aged for 24 hours in medium without any supplementation. A significantly higher proportion of embryos reached the blastocyst stage. However, this ratio still did not reach the ratio of blastocysts observed in the control group, in which the oocytes were parthenogenetically activated in MII without exposure to the aging process. Higher concentrations of H_2S donor (300 μM) did not have this positive effect (Table 2).


A
phosphorylated histone H1 signal intensity (%)
120
100
80
60
40
20
0
a,b
a
b
a
a
a
MII
C
Na2S
3Ki
C
Na2S
3Ki
A12
A24

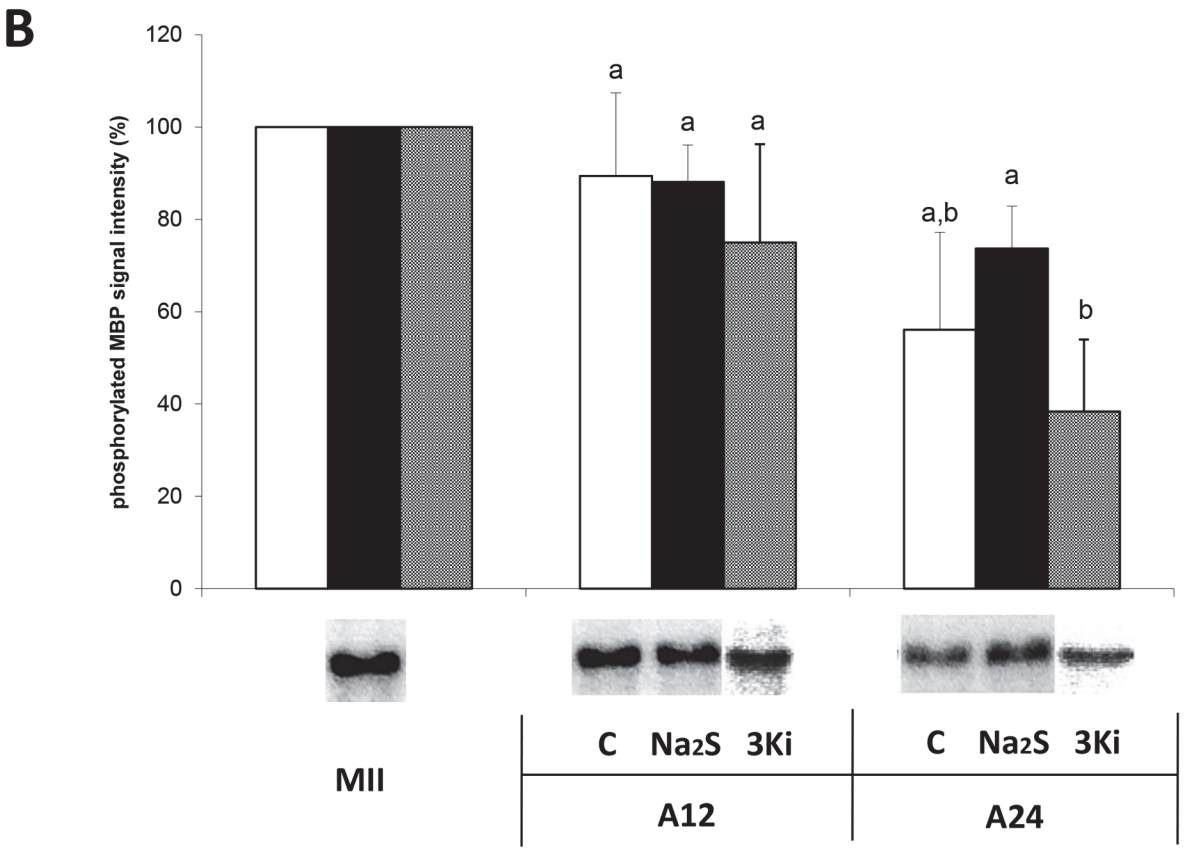

B
phosphorylated MBP signal intensity (%)
120
100
80
60
40
20
0
a
a
a
a,b
a
b
MII
C
Na2S
3Ki
C
Na2S
3Ki
A12
A24

Figure 6. Effect of H_2S donor on MPF and MAPK activity. 6A Histone H1 kinase assay was carried out to determine the activity of MPF by measurement of MPF capacity to phosphorylate its substrate (histone H1). **6B**: MBP kinase assay was carried out to determine the activity of MAPK by measurement of MAPK capacity to phosphorylate its substrate (MBP – Myelin basic protein). MPF and MAPK activities were determined in the MII oocytes (C – control, white column), the oocytes aged 12h and 24h in modified M199 medium, the oocytes aged 12h and 24h in modified M199 medium supplemented with a H_2S donor (Na_2S, black column), and the oocytes aged 12h and 24h in modified M199 medium supplemented with triple combination of inhibitors (3Ki, grey column). The results are presented as a ratio relative to the group of oocytes at metaphase II. *(GV – germinal vesicle stage; MII – oocytes at metaphase II; A12–12 hours of aging; A24–24 hours of aging; C – control, white column; Na_2S—$Na_2S.9H_2O$, 300 μM, black column; 3Ki - 1mM oxamic acid + 1mM beta-kyano-L-alanine + 5mM alpha-ketoglutaric acid disodium salt dihydrate).* ^{a,b,} *Statistically significant differences in activity (MPF or MAPK) between individual treatments at the same time are indicated with different superscripts ($P<0.05$).*

doi:10.1371/journal.pone.0116964.g006

Discussion

Although the gasotransmitter hydrogen sulfide has been recognized as a signal mediator, little is known about its involvement in oocyte physiology. In this study, we confirmed the role of hydrogen sulfide during the process of porcine oocyte aging in *in vitro* conditions. In a previous study, we demonstrated an effect of exogenous hydrogen sulfide (H_2S) on the meiotic maturation of porcine oocytes, emphasizing that H_2S could regulate oocyte meiosis as well as cumulus expansion [22]. The enzymes CBS, CSE and MPST are responsible for the endogenous production of H_2S in somatic cells [32,33]. Although these enzymes have not yet been detected in mammalian oocytes, one can argue that in porcine oocytes, these enzymes are clearly active based on our results showing the formation of endogenous H_2S and changes in its levels. This is the first evidence of endogenous H_2S in porcine oocytes. In addition, we also demonstrated a reduction of endogenous H_2S production as early as after 24 hours of the aging process of oocytes in *in vitro* conditions. Based on previous experiments, it is well known that, after this time point, morphological signs of oocyte aging are spontaneous parthenogenetic activation and, subsequently, the fragmentation or lysis of the oocytes [6].

Reduced H_2S production may be either one of the biochemical markers accompanying aging or a hallmark of the aging process. The role of H_2S in modulation of apoptosis on different somatic cells like lymphocytes, smooth muscle cells or fibroblast has been described [26]. Inhibition of endogenous H_2S production leads to apoptosis, and sodium hydrosulfide as an exogenous donor of H_2S can prevent apoptosis by improving mitochondrial dysfunction and suppressing the caspase-3 signaling pathway [34]. However, there is no evidence regarding the effect of H_2S in mammalian oocytes.

Table 1. Parthenogenetic activation of oocytes aged under the effect of the H_2S donor.

Groups	Na_2S	Activated oocytes (%)
MII	**0**	**94.2 ± 5.2^a**
24 hours	**0**	**79.2 ± 3.8^b**
24 hours	**150 μM**	**68.3 ± 3.8^b**
24 hours	**300 μM**	**42.5 ± 5.0^c**

At Oocytes were cultivated 48 hours to the metaphase II and then divided into 4 groups (see table). Control group (MII) was parthenogenetically activated immediately (without any exposure to prolonged cultivation). Other groups were exposed to prolonged cultivation (aging) for 24 hours in modified M199 medium supplemented with a H2S donor ($Na_2S.9H_2O$; 0μM, 150μM, and 300μM) and then parthenogenetically activated with calcium ionophore (25μM, 5 min) combined with 6-dimethyl aminopurine (2mM, 2 h). Subsequently, oocytes were cultured in NCSU 23 medium for the following 24 hours.
^{a,b,c} Statistically significant differences in the ratio of activated oocytes between individual treatments (in columns) are indicated with different superscripts ($P<0.05$).

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Table 2. Early embryonic development of parthenogenetically activated oocytes aged under the effect of the H_2S donor.

Groups	Na_2S	Cleavage (%)	Morula (%)	Blastocyst (%)
MII	0	76.7 ± 3.8[a]	26.7 ± 2.9[a]	24.2± 1.4[a]
24 hours	0	41.7 ± 2.9[c]	8.3 ± 1.4[b]	1.7 ± 1.4[c]
24 hours	150 μM	54.2 ± 3.8[b]	20.8 ± 3.8[a]	15.8 ± 2.9[b]
24 hours	300 μM	26.7 ± 2.4[d]	13.3 ± 2.4[b]	3.3 ± 1.2[c]

Oocytes were cultivated 48 hours to the metaphase II and then divided into 3 groups (see table). Control group (MII) was parthenogenetically activated immediately (without any exposure to prolonged cultivation). Other groups were exposed to prolonged cultivation (aging) for 24 hours in modified M199 medium supplemented with a H2S donor ($Na_2S.9H_2O$; 0μM, 150μM, and 300μM) and then parthenogenetically activated with calcium ionophore (25 μM, 5 min) combined with 6-dimethyl aminopurine (2 mM, 2 h). Subsequently, oocytes were cultured in NCSU 23 medium for 168 hours (7 days). The ratio of cleaved embryos was evaluated after the first 48 hours of culture.

[a,b,c,d] Statistically significant differences in type of embryo stage between individual treatments (in columns) are indicated with different superscripts ($P<0.05$).

doi:10.1371/journal.pone.0116964.t002

We tested the hypothesis that H_2S can play a role in aging by two means: first by sustaining and amplifying the decrease in H_2S production; second, by artificially increasing H_2S levels through the use of a hydrogen sulfide donor (Na_2S).

Oxamic acid, beta-kyano-L-alanine and alpha-ketoglutaric acid disodium salt dihydrate were used as specific inhibitors of the activities of CBS, CSE and MPST [35, 36]. The use of these inhibitors led to an acceleration of porcine oocyte aging and significantly increased the proportion of fragmented oocytes during aging. The enzymes CBS and CSE are expected to be present in porcine oocyte based on the effects of their inhibition. These enzymes are major participants in the active formation of H_2S in the reproductive tract [16], similarly to MPST, which has not yet been observed in the reproductive system of mammals.

It can be assumed that the increased proportion of apoptotic aged oocytes is largely caused by the decrease in endogenous H_2S production because this effect can be reversed. Indeed, in the presence of H_2S donor, a reversion of the negative effects of inhibitors was observed. Differing time courses of the aging process induced by CBS and CSE inhibition on the one hand and by MPST inhibition on the other, may reflect the specific roles of these enzymes in porcine oocytes or a distinct ability to compensate for the inhibition of one enzyme via other enzymes that were not affected by the specific inhibitors [37].

We also observed that addition of exogenous H_2S via a hydrogen sulfide donor resulted in the suppression of oocyte fragmentation, which is a manifestation of apoptosis [38]. The selected H_2S donor, Na_2S, effectively suppressed apoptosis in aged porcine oocytes at concentrations comparable to physiological concentrations of H_2S in tissues: the positive effect we observed at concentrations of $Na_2S.9H_2O$ above 150 μM and, for example, in brain and in other tissues, occurred at concentrations of H_2S that varied from 50 to 160 μM [14]. Based on these results, we hypothesize that the protective effect of an H_2S donor against oocyte fragmentation might be due to the compensation for the decrease in endogenous H_2S production that occurred spontaneously during the early stages of oocyte aging. Therefore, the influx of H_2S from exogenous resources may contribute to the suppression of manifestations of some signs of aging in oocyte.

Two hallmarks of aging in mammalian oocytes are the decline in the activity of both MPF (M-phase promoting factor) and MAPK (mitogen-activated protein kinase). If these declines are prevented, then the ratio of fragmented oocytes decreases [39,40,41]. In our study, we did not observe significant changes in the dynamics of either MPF or MAPK activity under the influence of H_2S donor at 12 and 24 hours of aging. The effect of an H_2S donor apparently occurs via other regulatory mechanisms that remain to be determined. Another gasotransmitter, NO,

appears to interfere with these pathways in an atypical manner. Jeseta et al. [42] described the parthenogenetic activation of *Xenopus laevis* oocytes in response to a nitric oxide donor, and this effect occurred in the absence of a dramatic effect on MPF activity. In the amphibian model, maintenance of MPF activity in metaphase-II-arrested oocytes has been shown to be required to trigger apoptosis [43]. Noticeably, a decrease in H_2S levels beyond those detected during aging led to a drop in MAPK kinase activity after 24 hours but did not impact MPF. Based on our observations, it can be speculated that the MPF and MAPK pathways exhibit different sensitivities to changes in H_2S changes that may be related to protein sulfhydration (e.g., MEK1, which is an activator of MAPK) [44]. Indeed, sulfhydration of proteins by H_2S significantly alters the activity of these proteins [37]. For example, the activity of pro-apoptotic and anti-apoptotic factors can vary significantly under the influence of H_2S [45]. Further studies are needed to characterize the members or regulators of these pathways that are modulated with respect to activities or location by sulfhydration.

Protection of oocytes against aging by increasing H_2S levels might incur a cost. Indeed, in our culture conditions, the aged oocytes exhibited a reduced response to calcium ionophore treatment, which normally induces parthenogenetic activation. This response was even lower in groups of oocytes that were aged in the presence of an H_2S donor. The causes of this decline are not yet known. Because parthenogenetic activation is a calcium-dependent process [46] and H_2S has been reported to modulate calcium ion channel activity [47], we can hypothesize that the presence of an H_2S donor impairs calcium signaling.

It is known that inadequate calcium signaling results in a reduced ratio of activated oocytes [48,49]. Surprisingly, however, a higher proportion of oocytes that had been aged in the presence of an H_2S donor developed to the blastocyst stage following activation with an ionophore. However, hydrogen sulfide expression has a positive effect because the proportion of blastocysts and the developmental competence are quality markers of oocytes that have matured *in vitro*. The survival and development of the embryo depends heavily on mRNAs and proteins that accumulated during the growth and maturation of the oocyte. A majority of these products are used during the first embryonic divisions, after which transcriptional activation occurs in the embryonic genome. During oocyte aging, a range of processes occur that significantly disrupt developmental competency [50]. H_2S appears to suppress at least some of these processes.

Although the intracellular mechanisms underlying the effect of H_2S on biochemical processes in aged oocytes remain to be fully deciphered, our study clearly demonstrated a role for H_2S in the protection of porcine oocytes against the aging process. Additional studies are needed to characterize the effects of H_2S on survival, fertilization and early developmental processes in porcine oocytes.

Supporting Information

S1 Table. Effects of an elevated H_2S level on porcine oocytes aging. Oocytes were cultivated to metaphase II and then exposed to prolonged cultivation in a modified M199 medium supplemented with a H_2S donor for next 72 hours ($Na_2S.9H_2O$; 0μM, 75μM, 150μM, 300 μM, and 600 μM). ^{a,b,c} *Statistically signifficant differences in type of oocytes between individual concentrations of hydrogen sulfide donor (in columns) are indicated with different superscripts ($P<0.05$). The total number of oocytes in each experimental group was 120.*
(DOCX)

S2 Table. Effects of an elevated H_2S level and inhibition of H_2S producing enzymes during oocyte aging - 24 hours. Oocytes were cultivated to metaphase II and then exposed to prolonged cultivation in a modified M199 medium for 24 hours in the presence of a H_2S donor or H_2S producing enzymes inhibitors. Na_2S ($Na_2S.9H_2O$; 300 μM) was used as the H_2S donor,

oxamic acid (1mM, OA) was used as a CBS inhibitor, beta-kyano-L-alanine (1mM, KA) was used as a CSE inhibitor and alpha-ketoglutaric acid disodium salt dihydrate (5mM, KGA) was used as a MPST inhibitor. ^{a,b,c,d} *Statistically signifficant differences in type of oocytes between individual treatments (in columns) are indicated with different superscripts (P<0.05). The total number of oocytes in each experimental group was 120.*
(DOCX)

S3 Table. Effects of an elevated H_2S level and inhibition of H_2S producing enzymes during oocyte aging - 48 hours. Oocytes were cultivated to metaphase II and then exposed to prolonged cultivation in a modified M199 medium for 48 hours in the presence of a H_2S donor or H_2S producing enzymes inhibitors. Na_2S ($Na_2S.9H_2O$; 300 μM) was used as the H_2S donor, oxamic acid (1mM, OA) was used as a CBS inhibitor, beta-kyano-L-alanine (1mM, KA) was used as a CSE inhibitor and alpha-ketoglutaric acid disodium salt dihydrate (5mM, KGA) was used as a MPST inhibitor. ^{a,b,c,d} *Statistically signifficant differences in type of oocytes between individual treatments (in columns) are indicated with different superscripts (P<0.05). The total number of oocytes in each experimental group was 120.*
(DOCX)

S4 Table. Effects of an elevated H_2S level and inhibition of H_2S producing enzymes during oocyte aging - 72 hours. Oocytes were cultivated to metaphase II and then exposed to prolonged cultivation in a modified M199 medium for 72 hours in the presence of a H_2S donor or H_2S producing enzymes inhibitors. Na_2S ($Na_2S.9H_2O$; 300 μM) was used as the H_2S donor, oxamic acid (1mM, OA) was used as a CBS inhibitor, beta-kyano-L-alanine (1mM, KA) was used as a CSE inhibitor and alpha-ketoglutaric acid disodium salt dihydrate (5mM, KGA) was used as a MPST inhibitor. ^{a,b,c} *Statistically signifficant differences in type of oocytes between individual treatments (in columns) are indicated with different superscripts (P<0.05). The total number of oocytes in each experimental group was 120.*
(DOCX)

S5 Table. Reversion of the effects of CBS, CSE and MPST inhibitors using a H_2S donor. Oocytes were cultivated to metaphase II and then exposed to prolonged cultivation (24 hours) in a modified M199 medium supplemented with a H_2S donor ($Na_2S.9H_2O$; 300 μM) and the following individual inhibitors: oxamic acid (1mM, OA), beta-kyano-L-alanine (1mM, KA), alpha-ketoglutaric acid disodium salt dihydrate (5mM, KGA), and its combination (see Table). ^{a,b,c,d,e} *Statistically signifficant differences in type of oocytes between individual treatments (in columns) are indicated with different superscripts (P<0.05). The total number of oocytes in each experimental group was 120.*
(DOCX)

S6 Table. Effects of concurrent CBS, CSE and MPST inhibition and its reversion using a H_2S donor - 24 hours of ageing. Oocytes were cultivated to metaphase II and then exposed to prolonged cultivation in a modified M199 medium supplemented with a H_2S donor ($Na_2S.9H_2O$; 300 μM) and the inhibitors for 24 hours. Various combinations of oxamic acid (1mM, OA) which was used as a CBS inhibitor, beta-kyano-L-alanine (1mM, KA) which was used as a CSE inhibitor and alpha-ketoglutaric acid disodium salt dihydrate (5mM, KGA) which was used as a MPST inhibitor were used in this experiment. To reverse effects of inhibitors, a H_2S donor (300 μM, $Na_2S.9H_2O$) was added to each experimental group. ^{a,b,c} *Statistically signifficant differences in type of oocytes between individual treatments (in columns) are indicated with different superscripts (P<0.05). The total number of oocytes in each experimental group was 120.*
(DOCX)

S7 Table. Effects of concurrent CBS, CSE and MPST inhibition and its reversion using a H_2S donor - 48 hours of ageing. Oocytes were cultivated to metaphase II and then exposed to prolonged cultivation in a modified M199 medium supplemented with a H_2S donor ($Na_2S.9H_2O$; 300 μM) and the inhibitors for 48 hours. Various combinations of oxamic acid (1mM, OA) which was used as a CBS inhibitor, beta-kyano-L-alanine (1mM, KA) which was used as a CSE inhibitor and alpha-ketoglutaric acid disodium salt dihydrate (5mM, KGA) which was used as a MPST inhibitor were used in this experiment. To reverse effects of inhibitors, a H_2S donor (300 μM, $Na_2S.9H_2O$) was added to each experimental group. ^{a,b,c} *Statistically signifficant differences in type of oocytes between individual treatments (in columns) are indicated with different superscripts ($P<0.05$). The total number of oocytes in each experimental group was 120.*
(DOCX)

S8 Table. Effects of concurrent CBS, CSE and MPST inhibition and its reversion using a H_2S donor - 72 hours of ageing. Oocytes were cultivated to metaphase II and then exposed to prolonged cultivation in a modified M199 medium supplemented with a H_2S donor ($Na_2S.9H_2O$; 300 μM) and the inhibitors for 72 hours. Various combinations of oxamic acid (1mM, OA) which was used as a CBS inhibitor, beta-kyano-L-alanine (1mM, KA) which was used as a CSE inhibitor and alpha-ketoglutaric acid disodium salt dihydrate (5mM, KGA) which was used as a MPST inhibitor were used in this experiment. To reverse effects of inhibitors, a H_2S donor (300 μM, $Na_2S.9H_2O$) was added to each experimental group. ^{a,b,c} *Statistically signifficant differences in type of oocytes between individual treatments (in columns) are indicated with different superscripts ($P<0.05$). The total number of oocytes in each experimental group was 120.*
(DOCX)

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Conceived and designed the experiments: TK M. Smelcova JP J-FB M. Sedmikova. Performed the experiments: TK M. Sedmikova JP JN MD AV IW VKC EC LT. Analyzed the data: TK JN MD IW VKC. Wrote the paper: TK JP M. Sedmikova J-FB FJ.

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5.3 Vliv suplementace kultivačního média česnekovými deriváty na meiotické zrání prasečích oocytů a embryonální vývoj

Česnekové deriváty slouží v řadě typů tkání jako donory gasotransmiteru sulfanu. Stanovená hypotéza, že suplementace kultivačního média česnekovými deriváty může zvýšit kvalitu oocytů kultivovaných v *in vitro* podmínkách, byla ověřena doplněním alliinu v koncentracích 0,05 a 0,1 mM a S-allyl cysteinu v koncentracích 0,1 – 1 mM do kultivačního média. Suplementace média česnekovými deriváty nepřinesla očekávaný efekt. Alliin narušil průběh jaderného zrání a S-allyl cystein neměl vliv na meiotické zrání oocytů, ale způsobil dřívější nástup rýhování po partenogenetické aktivaci a snížení hladin ROS ve zrajících a partenogeneticky aktivovaných oocytech.

Vliv česnekových derivátů na jaderné zrání byl hodnocen morfologickým hodnocením zrajících oocytů. Suplementace kultivačního média alliinem způsobila pokles podílu oocytů, které prodělaly GVBD po 20 hodinách kultivace (viz Příloha 1), a pokles podílu oocytů, které prodělaly přechod z meiózy I do meiózy II po 30 hodinách kultivace (viz Příloha 2). Tyto výsledky nebyly publikovány a účinky česnekového derivátu alliinu nebyly dále vyšetřovány.

Suplementace kultivačního média S-allyl cysteinem neměla vliv na jaderné zrání oocytů, ani na další markery meiotického zrání, kterými jsou průběh cytoplazmatického zrání a kumulární expanze.

Oocyty, které zrály v přítomnosti S-allyl cysteinu se ale ve zvýšené míře rýhovaly ve srovnání s oocyty dozrálými v médiu bez S-allyl cysteinu 22 hodin po partenogenetické aktivaci (41,67 – 43,33 % vs. 8,33 %).

Možným mechanismem, kterým S-allyl cystein působí na oocyty jeho jeho schopnost snižovat produkci ROS v buňkách. S-allyl cystein snížil produkci ROS ve zrajících oocytech o 82,9 – 91,6 % po 24 hodinách kultivace a o 86,4 – 99,1 % po 48 hodinách kultivace. Oocyty dozrálé v přítomnosti SAC a následně partenogeneticky aktivované a kultivované dalších 22 hodin v médiu bez přídavku SAC si rovněž udržely snížené hladiny ROS (o 57,8 – 66,3 %).

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The antioxidative properties of S-allyl cysteine not only influence somatic cells but also improve early embryo cleavage in pigs

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ABSTRACT

In vitro cultivation systems for oocytes and embryos are characterised by increased levels of reactive oxygen species (ROS), which can be balanced by the addition of suitable antioxidants. S-allyl cysteine (SAC) is a sulfur compound naturally occurring in garlic (*Allium sativum*), which is responsible for its high antioxidant properties. In this study, we demonstrated the capacity of SAC (0.1, 0.5 and 1.0 mM) to reduce levels of ROS in maturing oocytes significantly after 24 (reduced by 90.33, 82.87 and 91.62%, respectively) and 48 h (reduced by 86.35, 94.42 and 99.05%, respectively) cultivation, without leading to a disturbance of the standard course of meiotic maturation. Oocytes matured in the presence of SAC furthermore maintained reduced levels of ROS even 22 h after parthenogenic activation (reduced by 66.33, 61.64 and 57.80%, respectively). In these oocytes we also demonstrated a growth of early embryo cleavage rate (increased by 33.34, 35.00 and 35.00%, respectively). SAC may be a valuable supplement to cultivation media.

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INTRODUCTION

During meiotic maturation in in vitro conditions, oocytes acquire developmental competence, which is decisive with regard to the capacity of the fertilised oocyte to develop into a viable embryo (*Wassarman, 1988*). Oxidative stress negatively influences meiotic maturation by influencing the properties of its cytoskeleton. It damages the microfilaments of the maturing oocyte (*Jiao et al., 2013*), disturbs the dynamics of the microtubular network and the attachment of chromosomes to microtubules (*Choi et al., 2007*). Oxidative stress negatively influences homeostasis of calcium ions (*Ambruosi et al., 2011*). It also impairs the redistribution of cortical granules during the course of meiotic maturation, which increases the incidence of polyspermy after in vitro fertilisation (IVF) (*Jiao et al., 2013*). Excessive production of reactive oxygen species

(ROS) reduces the percentage of formed pronuclei in porcine oocytes following IVF (*Alvarez et al., 2015*).

Oxidative stress is a consequence of increased levels of ROS in cells. Balanced levels of ROS are important for the correct functioning of the organism, and are also important in the process of meiotic maturation. A proportionate amount of ROS in the follicular fluid supports germinal vesicle breakdown, by which the process of meiotic maturation begins (*Takami et al., 1999*). The follicular fluid also contains antioxidants. Balance of the levels of ROS and antioxidants in the follicular fluid is of key importance for the successful course of meiotic maturation (*Pasqualotto et al., 2004*).

In vitro cultivation systems used for the cultivation of oocytes are endangered by increased levels of ROS and the development of oxidative stress, because cultivation media contain a range of components manifesting pro-oxidative activity. These include, for example, energy sources such as lactate and pyruvate (*Hashimoto et al., 2000*), and hormones (*Markides, Roy & Liehr, 1998*). Transitory exposure of in vitro cultures to light (*Takenaka, Horiuchi & Yanagimachi, 2007*) and increased concentrations of oxygen (*Agarwal, Saleh & Bedaiwy, 2003*) also increases the production of ROS in cultivation systems.

Balancing increased levels of ROS in a cultivation medium by the addition of suitable antioxidants may prevent the development of oxidative stress and thus have a positive influence on early embryo cleavage of matured oocytes. Several sources of antioxidant substances are known, and more are being sought within the framework of ongoing studies. These, for example, include the amino acid cysteine, which reduces levels of ROS in maturing bovine oocytes (*Morado et al., 2009*). A cysteine derivative, N-acetyl cysteine (NAC), positively influences the formation of pronuclei and the development of blastocytes in vitro in pigs (*Whitaker, Casey & Taupier, 2012*).

Antioxidants also include a further cysteine derivative, the sulfur compound S-allyl cysteine (SAC), which is responsible for the high antioxidant activity of garlic (*Colín-González et al., 2015*). SAC is known for its anti-apoptotic and antioxidant effects in a range of types of somatic cells. SAC manifests antioxidant properties for example in the nervous (*Tsai et al., 2011*) and cardiovascular systems (*Louis et al., 2012*). *Takemura et al. (2014)* published a study demonstrating the antioxidant effects of SAC on rat sperm.

In somatic cells SAC manifests better antioxidant properties in comparison with cysteine. Upon oral administration to mice it brought about a larger increase in the activity of antioxidant enzymes in plasma, the kidneys and liver in comparison with cysteine (*Hsu et al., 2004*). In addition, according to *Dion, Agler & Milner (1997)*, SAC is more effective than cysteine in the protection of liver cells against the mutagenic effects of nitrosomorpholine. To date no study has been published dealing with the potential antioxidant effects of SAC on maturing oocytes.

The aim of the presented study was to test the hypothesis that SAC influences meiotic maturation of porcine oocytes and early embryo cleavage during in vitro cultivation.

MATERIALS AND METHODS

Collection and cultivation of oocytes and evaluation of meiotic maturation

Oocytes were obtained from ovaries through aspiration from follicles (2–5 mM in diameter) with 20G needles and cultured in a modified M199 medium (Gibco BRL, Life Technologies, Carlsbad, CA, USA) supplemented with calcium L-lactate (2.75 mM; Sigma Aldrich, USA), sodium pyruvate (0.25 mg/mL; Sigma Aldrich, USA), gentamicin (0.025 mg/mL; Sigma Aldrich, USA), HEPES (6.3 mM; Sigma Aldrich, USA), 10% (v/v) foetal calf serum (Gibco BRL, Life Technologies, Germany), albumin (5 mg/mL; Sigma Aldrich, USA) and 13.5 IU eCG: 6.6 IU hCG/mL (P.G. 600, Intravet, Boxmeer, Netherlands). Oocytes were cultured with SAC (Sigma Aldrich, USA) in concentrations of 0.0 (control), 0.1, 0.5, 1.0 and 5.0 mM for 24 and 48 h (39 °C; 5% CO_2). The concentration of 5.0 mM was applied in experiments concerning nuclear maturation only.

After culture oocytes were denuded of cumulus cells by repeated pipetting through a narrow glass capillary and mounted on slides. The following stages of meiotic maturation were evaluated under a phase contrast microscope: germinal vesicle (GV), metaphase I (MI) and metaphase II (MII).

MPF/MAPK double assay

Kinase Double Assay was performed according to *Kubelka et al. (2000)*. Briefly, samples were prepared from 15 oocytes cultivated with SAC by 5 μl extraction buffer addition and immediately frozen (−80 °C). Specific substrates H1 (Histone H1) and Myelin Basic Protein (MBP) were phosphorylated using radioactive labelled [γ-^{32}P]ATP, 500 μCi/mL (GE Healthcare Life Sciences, USA) and separated by SDS-PAGE. The signal intensities were measured by IP-plate, FLA 7000 reader (GE Healthcare Life Sciences, USA) and Multi-Gauge 2.0 software (Fujifilm, Japan). The obtained data was expressed relative to MPF/MAPK activities in oocytes in GV stage where we expect the lowest measured activities of MPF and MAPK.

Measurement of hyaluronic acid production within cumulus-oocyte complexes

Groups of 25 cumulus-oocyte complexes (COCs) were cultured for 24 and 48 h, washed four times in 500 μl PBS-PVA (0.01%) transfering them gently using a 50 μl pipette. Oocytes were denuded from cumulus cells and removed from samples. Samples were transferred into Eppendorf tubes, enzymatically digested using lyase from *Streptomyces hyalurolyticus* (20 μl/mL; Sigma-Aldrich, USA) at 39 °C overnight, centrifuged (5 min; 10,000 rpm, 4 °C) and measured in a Helios Epsilon spectrophotometer (Verkon, Czech Republic) at 216 nm.

Reactive oxygen species measurement

Reactive oxygen species production was evaluated in oocytes after 24 and 48 h of meiotic maturation and zygotes after 22 h of cultivation. Oocytes and zygotes were stained with 10 μM 2′,7′-dichlorodihydrofluorescin diacetate (Sigma-Aldrich, USA) (20 min; 39 °C) and mounted on glass. Samples were evaluated using a confocal microscope

(Leica SPE) and NIS Elements 4.0 software (Laboratory Imaging, Czech Republic). The results were expressed as the relative fluorescence intensity and related to the control group.

Parthenogenic activation of oocytes

Parthenogenic activation was carried out according to *Jílek et al. (2001)*. Briefly, matured oocytes denuded from cumulus cells were activated using calcium ionophore A23187 (25 μM, 5 min; Sigma Aldrich, USA) and 6-dimethylaminopurine—6-DMAP (2 mM, 2 h; Sigma Aldrich, USA) and cultivated in a modified M199 medium without hormones for 22 h. Activating potential was evaluated as the ratio of zygotes with 1 or 2 pronuclei and cleaving embryos. Early embryo cleavage was evaluated as the ratio of cleaving embryos among activated oocytes.

Statistical analysis

Each experimental group contained 120 oocytes for nuclear maturation and parthenogenic activation assessment, 100 for hyaluronic acid production assessment and 60 for MPF/MAPK Double Assay and ROS measurement. All experiments were repeated four times. SAS 9.0 Software (SAS Institute Inc., Cary, North Carolina, USA) was used for the statistical analyses. Significant differences between groups were determined using the one-way ANOVA test followed by Scheffe's method. $P < 0.05$ were considered significant. Statistically significant differences among different groups of oocytes are indicated by different superscripts.

Design of the experiments

Experiment 1 was performed in order to investigate the effect of SAC on the meiotic maturation of porcine oocytes. The oocytes were cultured for 24 and 48 h in vitro in the maturation medium described above, and supplemented with SAC in different concentrations. At the end of culture, stages of meiotic maturation, MPF and MAPK activity and hyaluronic acid production were evaluated.

Experiment 2 was focused on the effect of SAC on ROS levels in oocytes after 24 and 48 h of meiotic maturation and zygotes after subsequent 22 h of cultivation. We investigated the effect of SAC on ROS production as an indicator of oxidative stress and therefore quality of oocytes.

Experiment 3 was performed in order to evaluate the effect of SAC applied during meiotic maturation on subsequent parthenogenic activation. Activating potential and early embryo cleavage were considered as indicators of oocyte quality.

RESULTS

Effect of S-allyl cysteine on meiotic maturation of porcine oocytes

Nuclear maturation, cytoplasmic maturation and hyaluronic acid production were used as markers of successful meiotic maturation. Nuclear maturation was evaluated as a stage of meiotic maturation. SAC did not influence nuclear maturation despite the concentration of 5 mM which disrupts the standard course of the process (see Fig. 1A). This concentration was not applied in further experiments.

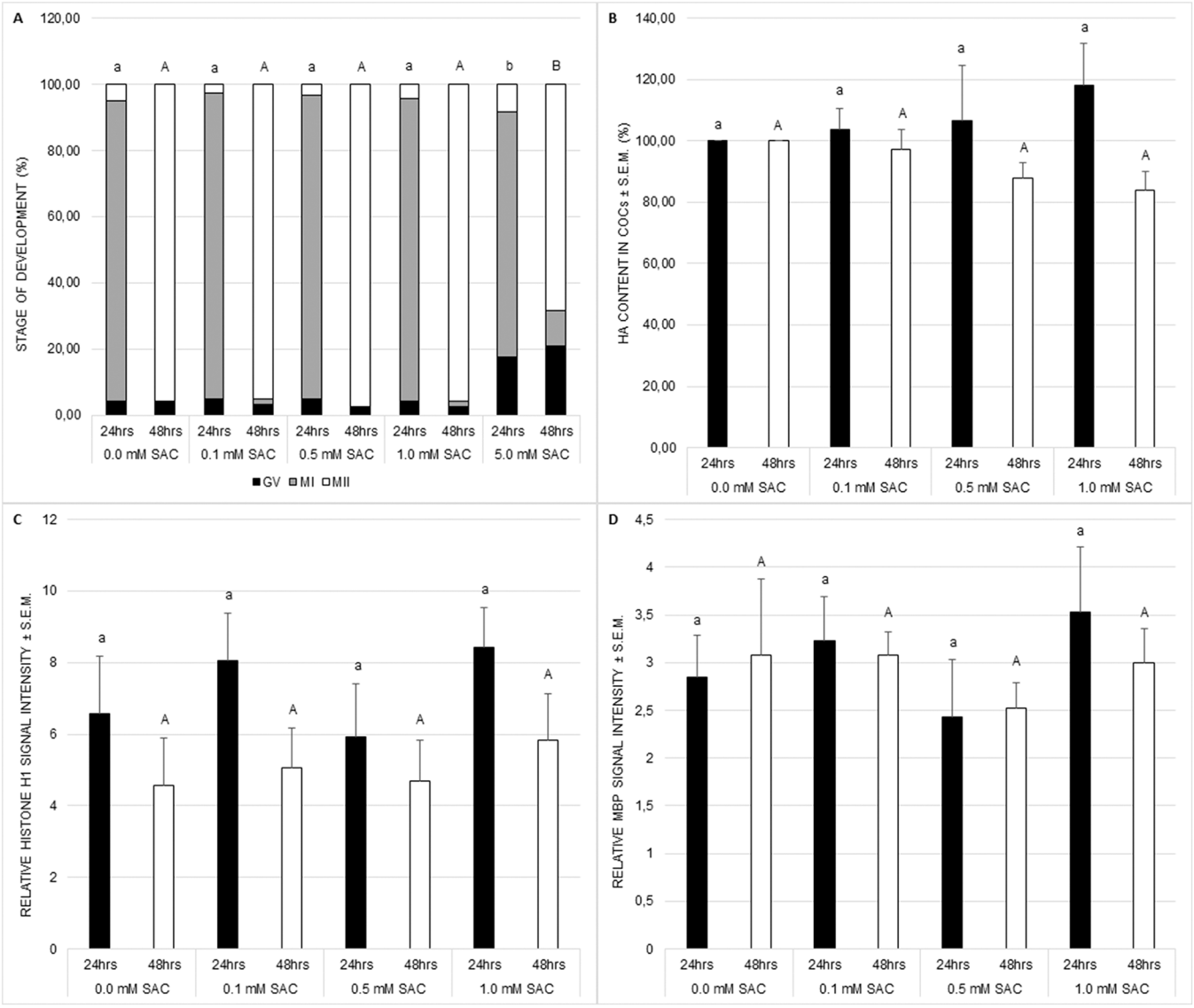

Figure 1 Effects of various SAC concentrations on porcine oocyte meiotic maturation after 24 and 48 h of cultivation. (A) Effects of SAC on nuclear maturation. GV–oocytes in the germinal vesicle stage, MI–oocytes in metaphase I, and MII–oocytes in metaphase II. Statistically significant differences between nuclear maturation stages (GV, MI, and MII) from various SAC concentrations are indicated by different superscripts: a, b–differences between nuclear maturation stages from various SAC concentrations after 24 h of cultivation ($P < 0.05$). A, B–differences between nuclear maturation stages from various SAC concentrations after 48 h of cultivation ($P < 0.05$). Data are presented as a mean of four replicates (n = 120 in each group). (B) Effects of SAC on hyaluronic acid (HA) content within COCs. Statistically significant differences between HA contents from various SAC concentrations are indicated by different superscripts: a, b–differences between HA contents from various SAC concentrations after 24 h of cultivation ($P < 0.05$). A, B–differences between HA contents from various SAC concentrations after 48 h of cultivation ($P < 0.05$). Data are presented as a mean ± S.E.M. of four replicates (n = 100 in each group). (C) Effects of SAC on MPF activity. Phosphorylated histone H1 signal intensity is related to signal intensity in GV oocytes and reflects changes in MPF activity. Statistically significant differences between relative histone H1 signal intensities from various SAC concentrations are indicated by different superscripts: a, b–differences between relative histone H1 signal intensities from various SAC concentrations after 24 h of cultivation ($P < 0.05$). A, B–differences between relative histone H1 signal intensities from various SAC concentrations after 48 h of cultivation ($P < 0.05$). Data are presented as a mean ± S.E.M. of four replicates (n = 60 in each group). (D) Effects of SAC on MAPK activity. Phosphorylated MBP signal intensity is related to signal intensity in GV oocytes and reflects changes in MAPK activity. Statistically significant differences between relative MBP signal intensities from various SAC concentrations are indicated by different superscripts: a, b–differences between relative MBP signal intensities from various SAC concentrations after 24 h of cultivation ($P < 0.05$). A, B–differences between relative MBP signal intensities from various SAC concentrations after 48 h of cultivation ($P < 0.05$). Data are presented as a mean ± S.E.M. of four replicates (n = 60 in each group).

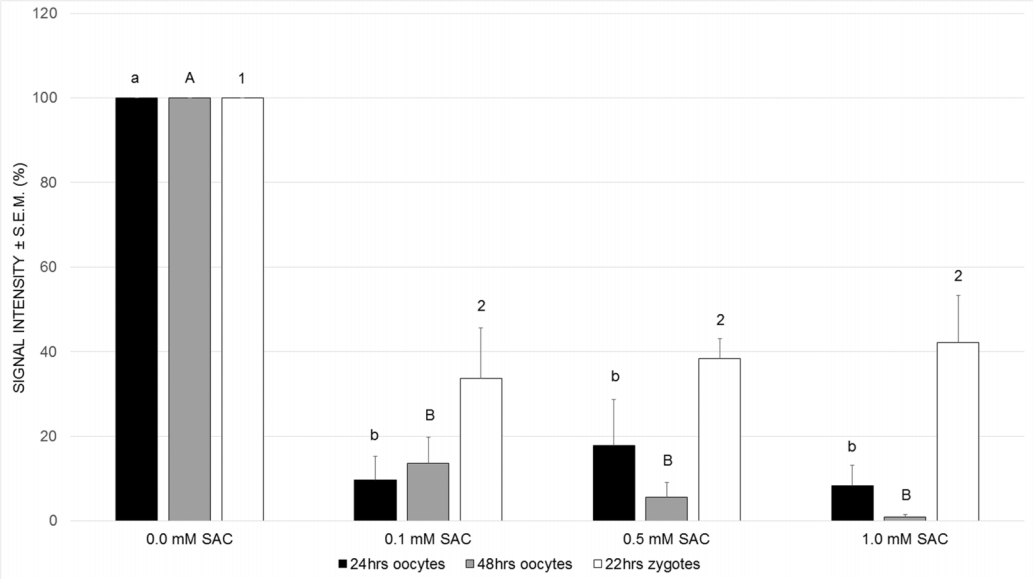

Figure 2 Effects of SAC on ROS production in porcine oocytes after 24 and 48 h of cultivation, and in zygotes after 22 h of cultivation. Statistically significant differences between ROS levels from various SAC concentrations are indicated by different superscripts: a, b–differences between ROS levels from various SAC concentrations after 24 h of cultivation ($P < 0.05$). A, B–differences between ROS levels from various SAC concentrations after 48 h of cultivation ($P < 0.05$). 1, 2–differences between ROS levels from various SAC concentrations after 22 h of cultivation of parthenogenetically activated oocytes ($P < 0.05$). Data are presented as a mean ± S.E.M. of four replicates (n = 60 in each group).

Cytoplasmic maturation was evaluated as MPF and MAPK activities. MPF and MAPK activities as well as hyaluronic acid production by COCs were not influenced by SAC (see Figs. 1B–1D).

Effect of S-allyl cysteine on reactive oxygen species production in porcine oocytes and zygotes

In these experiments we measured levels of ROS in order to prove our hypothesis that SAC has antioxidant activity in oocytes, as has been proven in somatic cells.

Primarily, we cultivated maturing oocytes in the presence of SAC in concentrations 0.1, 0.5 and 1.0 mM and evaluated levels of ROS within oocytes after 24 and 48 h of cultivation. We observed a significant decrease in ROS production in all experimental groups after 24 as well as 48 h of cultivation (see Fig. 2).

Obtaining these results, we continued in experiments by parthenogenic activation of oocytes matured in the presence of SAC. We evaluated ROS levels in zygotes 22 h after parthenogenic activation. According to our results, parthenogenetically activated zygotes maintained their antioxidant capacity and exhibited lowered ROS levels when compared to the control group (see Fig. 2).

Effect of S-allyl cysteine on oocyte parthenogenic activation

In the following experiments we focused on the effect of SAC on activating potential and early embryonic cleavage as indicators of embryo quality. SAC in concentrations

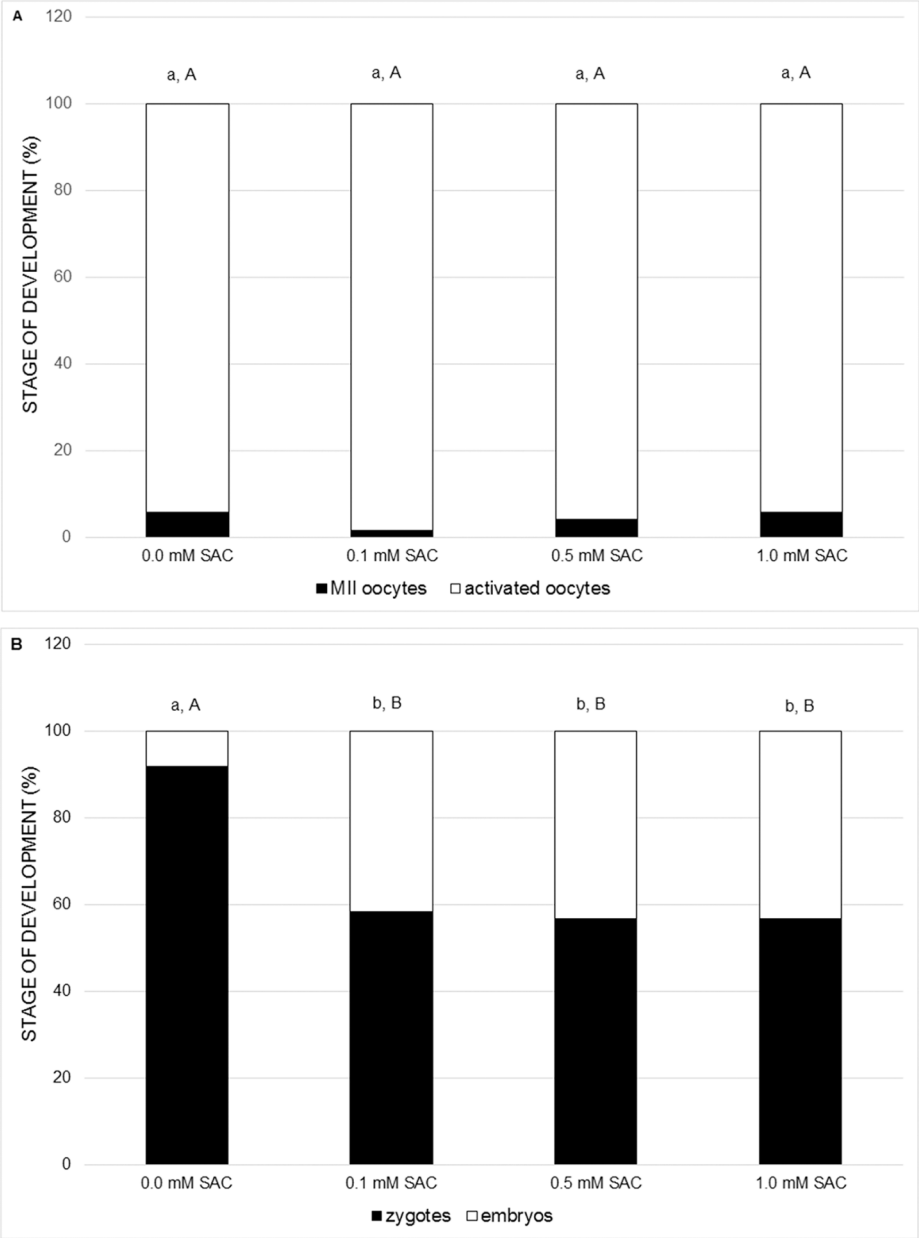

Figure 3 Effects of various SAC concentrations on parthenogenic activation of porcine oocytes. (A) Effects of SAC on the activating potential of parthenogenetically activated oocytes after 22 h of cultivation. MII oocytes–oocytes in metaphase II, activated oocytes–zygotes with one or two pronuclei and 2–3-cell cleaving embryos. Statistically significant differences between oocyte developmental stages from various SAC concentrations are indicated by different superscripts: a, b–differences between percentages of MII oocytes from various SAC concentrations ($P < 0.05$). A, B–differences between percentages of activated oocytes from various SAC concentrations ($P < 0.05$). Data are presented as a mean of four replicates (n = 120 in each group). (B) Effects of SAC on the early embryo development of parthenogenetically activated oocytes after 22 h of cultivation. Zygotes had one or two pronuclei; embryos consisted of 2 or 3 cells. Statistically significant differences between oocyte developmental stages from various SAC concentrations are indicated by different superscripts: a, b–differences between percentages of zygotes from various SAC concentrations ($P < 0.05$). A, B–differences between percentages of embryos from various SAC concentrations ($P < 0.05$). Data are presented as a mean of four replicates (n = 120 in each group).

0.1, 0.5 and 1.0 mM did not affect activating potential, however it enhances early embryo cleavage in comparison to the control (see Figs. 3A and 3B).

DISCUSSION

In our study, we demonstrated that SAC reduces levels of ROS in porcine oocytes during their maturation in vitro. In the case of oocytes maturing in the presence of SAC we did not observe deviations during the course of nuclear maturation or in the activity of kinases of key importance for the meiotic maturation of oocytes. Expansion of cumulus was also not influenced by cultivation. After parthenogenic activation we observed a higher proportion of cleaving embryos in oocytes maturing in the presence of SAC. The capacity of SAC to reduce intracellular levels of ROS has been described in somatic cells (*Tsai et al., 2011*). To the best of our knowledge, our study is the first to describe this effect of SAC on in vitro maturing mammal oocytes.

The marked reduction of intracellular levels of ROS observed in our study in porcine oocytes maturing in vitro in a medium enriched with SAC can be explained by the fact that both cysteine and the allyl group have antioxidant properties (*Chung, 2006*). It is known that the addition of cysteine alone or its derivatives (e.g. NAC) to the cultivation medium is capable of achieving a suppression of intracellular levels of ROS upon cultivation of oocytes and embryos in vitro (*Alvarez et al., 2015*; *Giorgi et al., 2015*). According to several in vivo experiments SAC has stronger antioxidant effects on various types of tissues than cysteine alone (*Hsu et al., 2004*) or than NAC (*Mizuguchi et al., 2006*).

In our study, the reduction of ROS levels did not have a significant impact on the observed aspects of maturation of porcine oocytes. This could indicate that porcine oocytes are relatively resistant to the effects of ROS. This is attested to also by the observations of *Alvarez et al. (2015)*, in which the increase of ROS levels had no impact on maturation. However, *Alvarez et al. (2015)* describe an increase in the proportion of oocytes maturing to metaphase II after a reduction of ROS by the addition of cysteine to the cultivation medium for maturation.

In our experiments we did not demonstrate the influence of SAC added during meiotic maturation on the proportion of oocytes emerging from metaphase II following parthenogenetic activation (thus the effect on the activation rate). However, in all applied concentrations (0.1, 0.5 and 1.0 mM), SAC increased the percentage of cleaving zygotes following parthenogenetic activation. A similar effect has also been described in the case of cysteine which, in the study by *Li et al. (2014)*, increased early embryo cleavage of porcine oocytes following ICSI, and also in the case of NAC, which improved the formation of male pronuclei and subsequent embryonic development (*Whitaker, Casey & Taupier, 2012*). On the basis of our results it is possible to conclude that SAC positively influences early embryo cleavage, a significant indicator of the quality of activated oocytes. This effect may be the result of suppression of ROS levels in zygotes, which persists from previous maturation of oocytes in the presence of SAC.

SAC need not act on oocytes cultivated in vitro only as an antioxidant reducing intracellular levels of ROS, but may also have an indirect effect via other target systems. SAC is also capable of increasing the activity of antioxidant enzymes such as catalase

and glutathione peroxidase (*Hsu et al., 2004*), by increasing intracellular levels of glutathione, which is known as a significant antioxidant responsible for uptake of ROS in cells (*Kohen & Nyska, 2002*). Also significant may be the capacity of SAC to increase the intracellular concentration of hydrogen sulfide (*Szabó, 2007*), which tanks among significant gaseous signalling molecules termed gasotransmitters (*Kamoun, 2004*). Hydrogen sulfide plays a significant role in regulating the maturation of mammal oocytes (*Nevoral et al., 2014*).

The extent to which hydrogen sulfide contributed to the effects of SAC we observed is not clear. In this study, in the case of COCs, after cultivation with SAC we did not observe an acceleration of maturation of oocytes or a suppression of expansion of cumulus cells, which is manifested under the influence of hydrogen sulfide on COCs (*Nevoral et al., 2014*). On the other hand, sulfide ions may have a whole range of indirect effects on oocytes. Hydrogen sulfide influences the activity of several proteins, including enzymes and the ion channels of their sulfhydration (*Paul & Snyder, 2012*). Sulfide ions also have an effect on the activity of other gasotransmitters—nitric oxide and carbon monoxide (*Li, Hsu & Moore, 2009*), which may significantly influence the maturation of oocytes (*Jablonka-Shariff & Olson, 1998*).

CONCLUSIONS

Further experiments will be required for a more detailed clarification of the effect of SAC on oocytes and their developmental competence. Our experiments indicate that SAC is an antioxidant suitable as a supplement to cultivation media for oocytes because it does not disturb the course of meiotic maturation, which is sensitive to imbalance of ROS. The addition of SAC to in vitro cultivation systems may make a significant contribution to the success of in vitro maturation and subsequent activation and the early embryonic development of oocytes.

ADDITIONAL INFORMATION AND DECLARATIONS

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Competing Interests

The authors declare that they have no competing interests.

Author Contributions

- Markéta Dvořáková conceived and designed the experiments, performed the experiments, analyzed the data, wrote the paper, prepared figures and/or tables.
- Ivona Heroutová performed the experiments.
- David Němeček performed the experiments.
- Kateřina Adámková performed the experiments.
- Tereza Krejčová performed the experiments.
- Jan Nevoral performed the experiments.
- Veronika Kučerová Chrpová performed the experiments.
- Jaroslav Petr conceived and designed the experiments, reviewed drafts of the paper.
- Markéta Sedmíková conceived and designed the experiments, reviewed drafts of the paper.

Data Deposition

The following information was supplied regarding data availability:

The raw data has been supplied as Supplemental Dataset Files.

Supplemental Information

Supplemental information for this article can be found online at http://dx.doi.org/10.7717/peerj.2280#supplemental-information.

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5.4 Účinky česnekových derivátů v organismu

Současné poznatky o metabolismu a účinku česnekových derivátů v organismu byly zpracovány a publikovány jako review: Dvořáková M., Weingartová I., Nevoral J., Němeček D., Krejčová T. (2015): Garlic sulfur compounds suppress cancerogenesis and oxidative stress: a review. Scientia Agriculturae Bohemica 46: 65-72.

GARLIC SULFUR COMPOUNDS SUPPRESS CANCEROGENESIS AND OXIDATIVE STRESS: A REVIEW*

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Garlic has long been considered a food with many health benefits. Several studies have confirmed that sulfur compounds are responsible for the positive effects of garlic on organisms. Garlic acts as an antioxidant by increasing antioxidant enzyme activity, reducing reactive oxygen species generation, and protecting proteins and lipids from oxidation. Garlic suppresses carcinogenesis through several mechanisms: (1) it reduces oxidative stress, and therefore, prevents damage to DNA; (2) it induces apoptosis or cell cycle arrest in cancer cells; and (3) it modifies gene expression through histon acetylation. The positive effects of garlic could be mediated by several mechanisms. It influences signalling pathways of gasotransmitters such as hydrogen sulfide. Garlic enhances hydrogen sulfide production both through its direct release and through an increase in activity of enzymes which produce hydrogen sulfide. Hydrogen sulfide acts as a signalling molecule in various tissues and participates in the regulation of many physiological processes. We can presume that garlic, which is able to release hydrogen sulfide, exhibits effects similar to those of this gasotransmitter.

cancer; diallyl disulfide; hydrogen sulfide; reactive oxygen species S-allyl cysteine

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INTRODUCTION

The first statements about the beneficial health effects of garlic originate from 2600–2100 BC. Ancient medical books from Greece, Rome, Egypt, India, and China recommended garlic consumption as a way to improve health (Gorinstein et al., 2007).

Garlic stimulates the immune system; it has antibacterial, antiviral, antiparasitic (Iciek et al., 2009), hepatoprotective (Banerjee et al., 2003), and neuroprotective properties (Borrelli et al., 2007), and decreases oxidative stress in cells. Garlic reduces the proliferation of some kinds of cancer cells (Banerjee et al., 2003). It lowers blood cholesterol and triacylglycerol levels, reduces blood pressure, and prevents the development of atherosclerosis. It impairs thromboxane synthesis and therefore acts as an anticoagulant (Iciek et al., 2009), and also decreases plasma glucose levels, which in turn prevents the development of diabetes mellitus (Banerjee et al., 2003).

Biosynthesis and metabolism of biologically active compounds from garlic

Garlic contains several sulfur compounds, which are known for their positive physiological effects. Interestingly, the effects of garlic differ depending on whether crushed fresh garlic or aged garlic extract are applied.

Whole garlic bulbs contain gamma-glutamyl cysteine, which undergoes two important reactions: (1) through hydrolysis and oxidation it is converted into S-alkenyl sulfoxides, which include alliin (S-allyl cysteine sulfoxide); (2) through gamma-glutamyl transpeptidase activity it is converted into S-allyl cysteine (SAC). This second reaction occurs during the long-term extraction of the garlic bulb (Corzo-Martinez et al., 2007).

Fresh garlic extract is formed when garlic bulbs are crushed. Alliin is converted into sulfonic acid, pyruvate, and ammonia during the process (Amagase,

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2006). The reaction is conditioned by the presence of vacuolar enzyme allinase. Whole alliin has been detected in the stomach, intestine, and liver of mice after its consumption without any further conversion (Egenschwind et al., 1992; Lachmann et al., 1994). The optimal pH level for allinase activity is 6.5; levels under 3.6 are typical for the stomach environment, in which alliinase is ineffective. Alliinase activity is completely and irreversibly inhibited in stomach acid (Lawson, Hughes, 1992). Heat-processed garlic contains mainly alliin because alliinase is destroyed at temperatures of 60°C and higher.

Sulfonic acid, created from alliin through allinase activity, is highly reactive and therefore it undergoes condensation, creating allicin (diallyl thiosulfinate) molecules (Lanzotti, 2006). Allicin is a volatile and highly unstable compound. It is instantly decomposed into oil-soluble diallyl monosulfide (DAS) and polysulfides, mainly diallyl disulfide (DADS) and diallyl trisulfide (DATS). Polysulfides are compounds likely to be responsible for the positive biological effects of garlic (Freeman, Kodera, 1995).

Allicin is also converted into ajoene, a compound considered responsible for the anticoagulant effects of garlic (Apitzcastro et al., 1983; Block et al., 1984).

Besides fresh garlic extract produced through the crushing of garlic bulbs, garlic could be processed through long-term extraction in 15–20% ethanol, which produces aged garlic extract (AGE). AGE contains less allicin in comparison with fresh garlic. Other compounds are generated during long-term extraction; gamma-glutamyl cysteine from intact garlic bulbs is converted into S-allyl cysteine (SAC) (Amagase, 2006; Colin-Gonzalez et al., 2012). SAC has been detected in blood after AGE consumption in a dose-dependent manner (Rosen et al., 2001; Steiner, Li, 2001).

Sulfur compounds from AGE are stable and odorless; they have a milder, less specific flavour. They exhibit greater and more stable positive effects within the organism. They are safer in comparison with raw garlic (Corzo-Martinez et al., 2007). Daily consumption of AGE at a dose of 1.8–10 g increases immune response in humans without any toxic side-effects (Amagase, 2006)

The metabolism of garlic compounds has yet to be fully understood. Garlic sulfur compound levels in blood after garlic consumption are good markers of garlic metabolism.

Garlic protects cells from oxidative stress

Oxidative stress is caused by an imbalance between free radical generation and endogenous antioxidative activity (Steare, Yellon, 1995). DNA, protein and lipid oxidation through reactive oxygen species (ROS) plays an important role in the ageing and development of many diseases. Endogenous antioxidants prevent cell damage through ROS. Reduced glutathione, superoxide dismutase, catalase, and glutathione peroxidase are among the most important endogenous antioxidants (Banerjee et al., 2003).

Regular consumption of garlic significantly increases cell antioxidative activity (Banerjee et al., 2002). Garlic prevents ROS generation and protects the mitochondrial membrane from oxidative stress, which alters membrane potential (Cervantes et al., 2013).

Alliin scavenges free radicals, protects lipids from oxidation, and increases antioxidative enzyme activity (Banerjee et al., 2003).

Fresh garlic extract scavenges free radicals in a dose-dependent manner (Prasad et al., 1996), and therefore it protects brain and heart cells from oxidative stress-induced ischemia (Batirel et al., 1996; Banerjee et al., 2003). Fresh garlic reduces lipid peroxidation in the heart, liver, and kidneys (Banerjee et al., 2001, 2002) and low-density lipoprotein (LDL) oxidation (Lau, 2001).

Allicin is the major compound of fresh garlic extract, however it is highly unstable. It reacts with cysteine and glutathione (Rabinkov et al., 2000), and creates substances which have antioxidative effects. Allicin and its derivatives can influence the activity of proteins through S-thiolation, which is one of many cell antioxidative mechanisms (Pinto et al., 2006). Allicin binds LDL, and therefore prevents LDL oxidation in blood-vessel walls (Gonen et al., 2005).

DAS and polysulfides increase the activity of antioxidative enzymes, and therefore protect cells from oxidative stress (Fukao et al., 2004).

AGE exhibits the highest antioxidative activity. It prevents damage to DNA by free radicals and therefore protects cells from cancer development (Borek, 2001). AGE reduces the risk of cardiovascular and cerebrovascular diseases (Lau et al., 1987). It prevents damage to cell membranes and lipid peroxidation in pulmonary endothelial cells subjected to oxidized LDL. AGE reduces hydrogen peroxide and superoxide production. It increases the activity of superoxide dismutase and glutathione peroxidase in pulmonary endothelial cells (Ide et al., 1997).

The antioxidative effects of garlic can be mediated by nitric oxide (NO) production. SAC, the main compound of AGE, regulates NO production by two different pathways:

Oxidative stress triggers nuclear factor kappa (NFκB) activity, which is involved in the expression of proinflammatory enzymes such as inducible nitric oxide synthase, producing NO in cells. SAC inhibits NFκB activity, and therefore it exhibits an antiinflammatory effect (Ide, Lau, 2001).

However, SAC can enhance NO production in cell through the activation of calcium-dependent nitric

oxide synthase. NO inhibits hydrogen peroxide and superoxide production. SAC increases NO production in endothelial cells, and therefore enhances blood-vessel elasticity and protects them from inflammation and development of atherosclerosis (Das et al., 1995; Sooranna et al., 1995).

Garlic inhibits carcinogenesis

Garlic inhibits the growth of some tumors and cell proliferation. DAS and DATS suppress benzo(a) pyren-induced development of stomach cancer in mice (Sparnins et al., 1988). DADS prevents intestine and kidney cancer in carcinogen-treated mice (Takahashi et al., 1992). Other studies confirm that garlic monosulfides and polysulfides are effective against *in vitro*-induced carcinogenesis (Wargovich et al., 1988; Schaffer et al., 1996; Suzui et al., 1997). Consumption of AGE at a dose of 2.4 ml daily for 12 months reduces the size and number of colorectal adenomas in humans (Tanaka et al., 2006). Garlic has an antiproliferative effect on human cancer cells in transgenetic animal models (Singh et al., 1996; Sundaram, Milner, 1996; Xiao et al., 2006a).

Considering the toxic effects of garlic sulfur compounds on cancer cells, it is necessary to investigate their potential toxic effects on normal cells. Certain studies demonstrate a higher tolerance of normal cells against the cytotoxic effects of garlic (Karmakar et al., 2007; Kim et al., 2007). However, other studies show toxic effects of garlic compounds on normal cells, especially those of the gastrointestinal tract (Joseph et al., 1989; Banerjee et al., 2003).

The anticarcinogenic effects of garlic compounds can be mediated by several mechanisms. Garlic induces an immune response in the organism (Lamm, Riggs, 2001). Garlic compounds prevent oxidative cell damage, and inhibit cell proliferation by induction of apoptosis or cell cycle arrest (Perchellet et al., 1990). They enhance the activity of detoxification enzymes, which improve excretion of carcinogens (Guyonnet et al., 1999), and suppress the activity of P450 enzymes, which mediate the activation of procarcinogens (Dion, Milner, 1997). Garlic has anticlastogenic effects and it contributes to repairing damaged DNA (Khanum et al., 2004). It influences gene expression through post-translational modifications, and therefore alters the activity of cell cycle regulating proteins (Druesne-Pecollo et al., 2007). It also suppresses blood supply to tumors. AGE prevents proliferation of endothelial cells and enhances their adhesion to collagen and fibronectin, which in turn reduces their mobility. AGE inhibits blood supply to human colorectal carcinoma through this mechanism (Matsuura et al., 2006). Alliin and DATS suppress angiogenesis by reducing vascular endothelial growth factor (VEGF) secretion (Mousa, Mousa, 2005).

Garlic induces apoptosis in cancer cells

Garlic polysulfides induce oxidative stress in cancer cells through increased production of ROS. Enhanced oxidative stress triggers apoptotic signalling pathway. Cancer cells are especially sensitive to garlic compounds, since they possess a small amount of molecules with antioxidative properties (Filomeni et al., 2003; Xiao et al., 2004).

DADS enhances ROS generation and therefore activates the c-Jun N-terminal kinase (JNK) pathway, which triggers cell death in neuroblastoma cells (Filomeni et al., 2003). DADS increases the expression of proapoptotic factors and reduces the expression of antiapoptotic factors in breast cancer cells (Nakagawa et al., 2001). It enhances intracellular levels of calcium ions, which in turn increases hydrogen peroxide production and activates caspases in human leukemic cells (Park et al., 2002).

DATS is more effective in the induction of apoptosis in human prostate cancer cells in comparison with DAS and DADS (Xiao et al., 2004). It degrades ferritin and therefore enhances cellular labile iron, which is in turn followed by ROS generation (Antosiewicz et al., 2006). It hyperphosphorylates and inactivates antiapoptotic factors through JNK and extracellular signal-regulated kinase 1/2 (ERK 1/2) signalling pathways (Xiao et al., 2004). It causes conformational changes of proapoptotic factors, which lead to their transfer into mitochondria (Kim et al., 2007).

Ajoene activates NFκB, stimulates ROS generation, induces apoptosis and cell cycle arrest in the G2 phase (Xu et al., 2004), and decreases intracellular levels of antiapoptotic factors (Li et al., 2002).

Garlic induces cell cycle arrest in cancer cells

A large amount of studies have confirmed the ability of garlic to induce cell cycle arrest, which could be a mechanism of cancerogenesis inhibition.

Garlic polysulfides inhibit cyclin-dependent kinase 1 (Cdk1) and enhance cyclin B1 expression in colon cancer cells (Knowles, Milner, 2000). They reduce Cdk-activating kinase activity in liver cancer cells (Wu et al., 2004) and Cdc25 activity in prostate cancer cells (Arunkumar et al., 2006). They activate mitogen-activated protein kinase (MAPK) p38, which reduces Cdc25 phosphatase activity in stomach cancer cells (Yuan et al., 2004). DATS is more effective in the induction of cell cycle arrest in the liver (Wu et al., 2004) and prostate cancer cells in comparison with DAS and DADS. Normal epithelial prostate cells are more resistant to the toxic effect of DADS than cancer cells (Xiao et al., 2005).

Garlic polysulfides can induce cell cycle arrest in the S phase, G2 phase or prometaphase. Cell cycle arrest in G2 is caused by a reduction in the cdc25 activity (Xiao et al., 2005). Cell cycle arrest in the

prometaphase is connected with hypermethylation of key subunits of the anaphase-promoting complex/cyclosome (APC/C) (Herman-Antosiewicz et al., 2007).

Garlic induces microtubule depolymerization and changes in cytoskeleton in interphase cancer cells and disrupts mitotic spindle formation in mitosis (Hosono et al., 2005; Zhang et al., 2006). Garlic polysulfides initiate changes in the microtubule network, chromatin condensation, and phosphorylation of histone H3 in the M phase (Herman-Antosiewicz, Singh, 2005). DATS induces oxidative changes in beta-tubulin cysteine residues *in vitro* (Hosono et al., 2005).

Garlic regulates gene expression through histone acetylation

Garlic compounds mediate histone acetylation through an increase of histone acetylase activity and a reduction of histone deacetylase activity, and can therefore alter gene expression. Histone modification influences the expression of proteins in cancer cells and expression of proteins involved in cell cycle regulation.

DADS induces histone acetylation in human breast cancer cells and rat liver cancer cells through a reduction of histone deacytylase activity (Lea et al., 1999), and affects the activity of cell cycle regulating proteins, such as p21 kinase, MAPK3, inhibitors of DNA-binding proteins, and proteins involved in DNA repair through histone modification (Druesne-Pecollo et al., 2007). DADS enhances histone H3 and H4 acetylation in human leukemic cells and inhibits histone deacetylases in liver and breast cancer cells (Lea et al., 1999). Allicin and SAC enhance histone acetylation in breast and intestine cancer cells (Lea et al., 2002).

Garlic influences cell signalization through hydrogen sulfide production

Hydrogen sulfide is well known as a toxic gas. In recent times, its physiological function as a gaseous signalling molecule, a gasotransmitter within the organism, has been discovered (Wang, 2002). Hydrogen sulfide is a signalling molecule in the nervous (Abe, Kimura, 1996), cardiovascular (Zhao et al., 2001) and reproductive systems (Srilatha et al., 2007). Cystathionine-gamma-lyase (CSE) and cystationine-beta-synthase (CBS) are enzymes synthesizing hydrogen sulfide within the organism (Wang, 2002).

Positive garlic effects can be mediated through hydrogen sulfide production. Human red blood cells produce hydrogen sulfide in anoxic conditions from garlic extract, allicin, DADS, and DATS in the presence of glutathione. The highest amount of hydrogen sulfide is generated from DATS. Addition of DADS into blood enhances amounts of exhaled hydrogen sulfide in rats (Insko et al., 2009). DADS and DATS vasodilatate blood-vessels through hydrogen sulfide production (Benavides et al., 2007).

Hydrogen sulfide can be generated from cysteine derivatives through the activity of cystathionine gamma-lyase (CSE) (Wang et al., 2010). One of these cysteine derivatives, SAC, can act as a direct substrate for hydrogen sulfide production, as well as a compound regulating CSE activity. SAC enhances CSE activity, and therefore protects heart cells against infarction (Chuah et al., 2007). S-propargyl cysteine (SPRC), a structural analog of SAC, enhances CSE expression. Created hydrogen sulfide enhances p53 and Bax expression. SPRC reduces the viability and division of cancer cells through this mechanism (Ma et al., 2011).

CONCLUSION

Sulfur compounds from garlic have a significant positive effect on organisms. The method of processing is a key factor determining the biological activity of garlic. Garlic could be administered as a fresh or aged garlic extract (AGE). Fresh garlic extract possesses both prooxidative and antioxidative properties, which depends on a type of treated tissue. Cancer represents one of the most investigated diseases in the developed world. Cancer cells exhibit high sensitivity to oxidative stress-induced apoptosis mediated by garlic compounds when compared to healthy cells. Many currently widespread diseases, such as cardiovascular diseases, are caused by high content of reactive oxygen species in the environment which could be balanced by garlic compounds, especially those contained in aged garlic extract. Hydrogen sulfide is a gaseous molecule exhibiting antioxidative activity. It stabilizes mitochondrial membranes, and therefore protects cells against apoptosis. Garlic sulfur compounds are able to release hydrogen sulfide, which could represent their mechanism of action. This presumption should be approved more thoroughly using different types of tissues for wider implication of garlic health benefits.

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Abbreviations:

AGE = aged garlic extract, CSE = cystathionine-gamma-lyase, DADS = diallyl disulfide, DAS = diallyl monosulfide, DATS = diallyl trisulfide, ERK = extracellular signal-regulated kinase, JNK = c-Jun N-terminal kinase, LDL = low-density lipoprotein, MAPK = mitogen-activated protein kinase, NFκB = nuclear factor kappa, ROS = reactive oxygen species, SAC = S-allyl cysteine, SPRC = S-propargyl cysteine, VEGF = vascular endothelial growth factor

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6 Diskuze

6.1 Vliv suplementace kultivačního média donorem sulfanu během meiotického zrání prasečích oocytů

Cílem experimentů bylo vyhodnotit vliv exogenního donoru sulfanu na průběh meiotického zrání a časný embryonální vývoj prasečích oocytů. Suplementace kultivačního média donorem sulfanu měla za následek zrychlený průběh jaderného zrání. Přítomnost donoru sulfanu v kultivačním médiu zároveň urychlila vzestup aktivity MPF a MAPK po 20 – 22 hodinách meiotického zrání, tedy v době časově korespondující s nástupem GVBD. Lze tak předpokládat, že zrychlený průběh jaderného zrání byl způsoben dřívějším vzestupem aktivity MPF a MAPK. Jakým způsobem sulfan ovlivnil aktivitu MPF a MAPK není známo. Sulfan ovlivňuje cílové proteiny mechanismem jejich sulfhydratace (Mustafa *et al.*, 2009), což může být i případ v naší studii, i když doposud nebyla publikována žádná práce, která by prokazovala právě přímou sulfhydrataci molekul MPF a MAPK sulfanem. Sulfan může také sulfhydratovat některé iontové kanály (Tang *et al.*, 2010) a další molekuly regulující aktivitu MPF a MAPK (Hu *et al.*, 2008) a ovlivňovat tak jejich aktivitu nepřímo. V somatických buňkách bylo pozorováno zapojení sulfanu do signálních drah cAMP a protein kinázy A (Njie-Mbye *et al.*, 2012) a fosfatidylinositol-3-kinázy a protein kinázy B (Huang *et al.*, 2010), které jsou zapojeny do kontroly kinázové aktivity také v savčím oocytu (Wassarman, 1988).

Donor sulfanu potlačil celkovou produkci hyaluronové kyseliny kumulo-oocytárními komplexy. Mechanismus, kterým sulfan působí na produkci hyaluronové kyseliny je dosud neznámý. Dříve již bylo prokázáno, že po dosažení metafáze I dochází k poklesu aktivity faktorů stimulujících produkci hyaluronové kyseliny (Nagyova *et al.*, 2000). Je možné, že donor sulfanu prohlubuje pokles aktivity těchto faktorů během meiózy II. Další možností je zapojení sulfanu do signální dráhy cAMP a protein kinázy A (Njie-Mbye *et al.*, 2012), která se podílí na regulaci procesu kumulární expanze (Eppig, 2001).

Pro účely vyhodnocení úlohy oocytu v procesu produkce hyaluronové kyseliny ovlivněné přítomností donoru sulfanu v kultivačním médiu byly kultivované oocytektomované komplexy. Oocytektomie sice snížila celkovou produkci hyaluronové kyseliny, ale vliv donoru sulfanu na ocytektomované komplexy nebyl prokázán. Snížení produkce HA při kultivaci ocytektomovaných komplexů lze vysvětlit tím, že proces kumulární expanze je z velké části regulován molekulami pocházejícími přímo z oocytu (Nagyova *et al.*, 2000; Eppig, 2001). Autoři Kimura *et al.* (2002) také pozorovali pokles exprese syntázy hyaluronové kyseliny 2 v kumulárních buňkách oocytektomovaných komplexů. Donor

sulfanu neměl vliv na produkci hyaluronové kyseliny oocytektomovanými komplexy, což naznačuje, že potlačení kumulární expanze donorem sulfanu je zprostředkováno samotným oocytem. Cílové molekuly donoru sulfanu v oocytu, které regulují produkci hyaluronové kyseliny, zůstávají neznámé. Může se jednat o proteiny rodiny transformujícího růstového faktoru β, které jsou cílem sulfanu v žaludečních buňkách potkana (Mard *et al.,* 2012) a regulují aktivitu syntázy hyaluronové kyseliny 2 v kumulárních buňkách myši (Dragovic *et al.,* 2005).

Oocyty, které zrály v přítomnosti donoru sulfanu, vykazovaly lepší aktivační potenciál. Mechanismus, kterým donor sulfanu ovlivnil aktivační potenciál oocytů, zůstává neznámý. Jak bylo prokázáno, donor sulfanu zasahuje během meiotického zrání do signálních drah MPF a MAPK, prostřednictvím kterých může ovlivnit také proces aktivace oocytu (Sanders *et* Swann, 2016). Pozitivní účinek na aktivační potenciál byl popsán i u jiného gasotransmiteru, oxidu dusnatého (Petr *et al.,* 2010).

6.2 Vliv suplementace kultivačního média donorem sulfanu během prodloužené kultivace prasečích oocytů

Cílem experimentů bylo vyhodnotit endogenní produkci sulfanu ve stárnoucích oocytech a vliv exogenního donoru sulfanu na průběh stárnutí prasečích oocytů a jejich časný embryonální vývoj. Bylo prokázáno, že ve stárnoucích prasečích oocytech dochází k endogenní produkci sulfanu prostřednictvím aktivity sulfan uvolňujících enzymů, a že tato endogenní produkce sulfanu klesá po 24 hodinách prodloužené kultivace. Fyziologická produkce sulfanu již byla prokázána v řadě typů buněk a tkání, například v játrech, ledvinách, mozku, hladké svalovině, slinivce a lymfocytech (Yang *et* Wang, 2007). Endogenní produkce sulfanu chrání řadu typů tkání před poškozením při patologických procesech. Například během ischémie myokardu dochází k endogenní produkci sulfanu, která chrání myokard před poškozením (Sivarajah *et al.,* 2006). Snížení endogenní produkce sulfanu bylo zaznamenáno při řadě onemocnění, jako jsou onemocnění kardiovaskulární soustavy, katarakta a jaterní cirhóza (Yang *et* Wang, 2007).

Suplementace kultivačního média donorem sulfanu zcela potlačila fragmentaci stárnoucích oocytů po 48 a 72 hodinách prodloužené kultivace. Lze předpokládat, že protektivní účinek donoru sulfanu je zprostředkován jeho schopností kompenzovat pokles endogenní produkce sulfanu, ke kterému dochází spontánně po prvních 24 hodinách prodloužené kultivace. Význam sulfanu v regulaci apoptózy byl prokázán v řadě typů somatických buněk. Donor

sulfanu působí antiapoptoticky v prasečích oocytech v koncentracích, které působí fyziologicky v jiných tkáních, například nervové soustavě (Elsey *et al.*, 2010). Ošetření kardiomyocytů potkana donorem sulfanu zvyšuje jejich přežitelnost po uměle vyvolané ischémii (Bian *et al.*, 2006). Donor sulfanu rovněž zmenšuje velikost tkáně postižené infarktem po uměle vyvolané ischémii a reperfuzi (Sivarajah *et al.*, 2006). Donor sulfanu zvyšuje přežitelnost neutrofilů s účinkem závislým na dávce (Rinaldi *et al.*, 2006).

Potlačení endogenní produkce sulfanu specifickými inhibitory sulfan uvolňujících enzymů vedlo k výraznému nárůstu podílu fragmentovaných oocytů. Obdobné účinky inhibice aktivity sulfan uvolňujících enzymů byly zaznamenány u somatických buněk. Ošetření srdeční tkáně potkana specifickými inhibitory CSE snížilo přežitelnost srdečních buněk po ischémii (Bian *et al.*, 2006). Inhibitor CSE způsobil rovněž rozsáhlejší poškození srdeční tkáně infarktem po uměle vyvolané ischémii a reperfuzi (Sivarajah *et al.*, 2006).

Kvalitu oocytů vystavených účinkům inhibitorů endogenní produkce sulfanu lze zlepšit exogenně dodaným donorem sulfanu. To je v souladu s výsledky publikovanými v práci (Li *et al.*, 2014a), kde inhibice endogenní produkce sulfanu vedla k apoptóze mezenchymálních kmenových buněk. I zde bylo prokázáno, že negativní vliv inhibice sulfan uvolňujících enzymů lze zvrátit dodáním exogenního donoru sulfanu. Donor sulfanu zlepšil jejich mitochondriální funkce a potlačil aktivitu kaspázy-3 (Li *et al.*, 2014a). Jiný průběh stárnutí u oocytů ošetřených jednotlivými inhibitory sulfan uvolňujících enzymů může vypovídat o rozdílné úloze jednotlivých enzymů v prasečím oocytu nebo o rozdílné schopnosti jednotlivých enzymů navzájem se zastupovat.

Přestože suplementace média donorem sulfanu a inhibitory sulfan uvolňujících enzymů ovlivnila výskyt morfologických projevů stárnutí oocytů, donor sulfanu a inhibitory sulfan uvolňujících enzymů neměly vliv na aktivitu MPF a MAPK během prodloužené kultivace oocytů. Donor sulfanu tak působí na stárnoucí prasečí oocyty pravděpodobně prostřednictvím jiného mechanismu, který je třeba objasnit.

Stárnutí oocytů v přítomnosti donoru sulfanu v koncentraci 0,3 mM, která působí pozitivně na průběh meiotického zrání a stárnutí oocytů, snižuje odpověď oocytů na ošetření ionoforem vápníku, který za normálních okolností navozuje partenogenetickou aktivaci. Sulfan ovlivňuje aktivitu Ca^{2+} iontových kanálů (Tang *et al.*, 2010) a partenogenetická aktivace oocytů je proces závislý na nitrobuněčné oscilaci Ca^{2+} iontů (Vitullo *et al.*, 1992), která může být přítomností donoru sulfanu v koncentraci 0,3 mM narušena. Narušení signalizace Ca^{2+} iontů vede ke snížení podílu aktivovaných oocytů (Kline *et al.*, 1992). Oocyty ošetřené donorem sulfanu v nižší koncentraci (0,15 mM) nicméně vykazovaly vyšší úspěšnost v dosažení stádií

moruly a blastocyty po sedmi dnech kultivace, což naznačuje zlepšení vývojové kompetence takto ošetřených stárnoucích oocytů.

6.3 Vliv suplementace kultivačního média česnekovými deriváty na meiotické zrání prasečích oocytů a embryonální vývoj

Cílem experimentů bylo vyhodnotit vliv česnekových derivátů na průběh meiotického zrání a časný embryonální vývoj prasečích oocytů. Účinky česnekových derivátů jsou v řadě typů tkání zprostředkovány jejich schopností zvyšovat koncentrace gasotransmiteru sulfanu (Chuah *et al.*, 2007; Mukherjee *et al*., 2009; Louis *et al.*, 2012). Suplementace kultivačního média česnekovými deriváty ale působila na zrající oocyty odlišným způsobem než suplementace kultivačního média donorem sulfanu. Přítomnost alliinu způsobila narušení průběhu jaderného zrání oocytů. Suplementace kultivačního média S-allyl cysteinem neměla vliv na jaderné zrání oocytů, ani na další markery meiotického zrání, kterými jsou průběh cytoplazmatického zrání a kumulární expanze. Alliin a z něj odvozené česnekové deriváty vykazují v organismu dvojí aktivitu, působí antiproliferativně a antioxidačně v závislosti na aplikované dávce a typu tkáně (Banerjee *et al.*, 2003; Mousa *et* Mousa, 2005; Izdebska *et al.*, 2016). Je tedy možné, že alliin v koncentraci 0,05 – 0,1 mM působí na zrající oocyty spíš cytostaticky. Naproti tomu vyzrálý česnekový extrakt, jehož hlavní složkou je S-allyl cystein, vykazuje v organismu vyšší a stálejší protektivní účinky než česnekové deriváty odvozené od alliinu (Corzo-Martinez *et al.*, 2007).

S-allyl cystein sice neměl vliv na markery meiotického zrání oocytů, ale pozitivně ovlivnil časný embryonální vývoj oocytů dozrálých v jeho přítomnosti. S-allyl cystein urychlil nástup rýhování zygot po partenogenetické aktivaci. Časný nástup rýhování přitom pozitivně koreluje s úspěšností následného embryonálního vývoje jak po IVF (Torner *et al.*, 2013), tak po partenogenetické aktivaci (Isom *et al.*, 2012) prasečích oocytů. Podobný efekt byl popsán u cysteinu, jehož je S-allyl cystein derivátem, který zvyšoval ve studii autorů Li *et al.* (2014b) podíl rýhujících se prasečích oocytů po ICSI, tak i u jiného derivátu cysteinu, N-acetyl cysteinu, který zlepšoval formaci samčích prvojader a následný embryonální vývoj (Whitaker *et al.*, 2012).

Při hledání mechanismu, kterým ovlivňuje S-allyl cystein oocyty jsme zjistili, že dochází k výraznému snížení hladin ROS ve zrajících oocytech ošetřených S-allyl cysteinem. Obdobný účinek SAC byl popsán u somatických buněk (Tsai *et al.*, 2011). Razantní snížení intracelulárních hladin ROS pozorované v naší studii lze vysvětlit skutečností, že antioxidační

účinky má jak cystein tak i allylová skupina (Chung, 2006). Je známo, že přídavkem samotného cysteinu nebo jeho derivátů, například N-acetyl cysteinu, do kultivačního média lze dosáhnout potlačení intarcelulárních hladin ROS při kultivaci oocytů a embryí *in vitro* (Alvarez *et al.*, 2015). V somatických buňkách přitom vykazuje S-allyl cystein silnější antioxidační účinky než cystein (Hsu *et al.*, 2004) a N-acetyl cystein (Mizuguchi *et al.*, 2006). Oocyty dozrálé v přítomnosti S-allyl cysteinu a následně partenogeneticky aktivované a kultivované v médiu bez přídavku S-allyl cysteinu si udržely snížené hladiny ROS. Je možné, že pozitivní účinek S-allyl cysteinu na nástup rýhování embryí je důsledkem potlačení hladin ROS v zygotách, které přetrvává z předchozího zrání oocytů v přítomnosti S-allyl cysteinu.

Jakým mechanismem S-allyl cystein snižuje hladiny ROS v oocytech a zygotách není známo V somatických buňkách je S-allyl cystein schopen zvyšovat aktivitu antioxidačních enzymů, jako jsou kataláza a glutation peroxidáza (Hsu *et al.*, 2004), a zvyšovat intracelulární hladiny glutationu, který je znám jako významný antioxidant zodpovědný za vychytávání ROS v buňkách (Kohen *et* Nyska, 2002). Významná může být také schopnost S-allyl cysteinu zvyšovat intracelulárního koncentrace sulfanu (Szabo, 2007), o kterém je známo, že působí v buňkách jako antioxidant (Mustafa *et al.*, 2009).

Nakolik se sulfan podílel na námi pozorovaných efektech S-allyl cysteinu, není jasné. Po kultivaci zrajících oocytů s S-allyl cysteinem nebyla pozorována ani akcelerace jaderného a cytoplazmatického zrání oocytů, ani potlačení expanze kumulárních buněk. Na druhé straně ale můžou sulfidové ionty působit na oocyty celou řadou nepřímých efektů. Sulfan ovlivňuje aktivitu mnoha proteinů včetně enzymů a iontových kanálů jejich sulfhydratací (Paul *et* Snyder, 2012). Sulfidové ionty také působí na aktivitu dalších gasotransmiterů, oxidu dusnatého a oxidu uhelnatého (Li *et al.*, 2009), které mohou významně ovlivnit zrání oocytů (Jablonka-Sharif *et* Olson, 1998).

7 Závěr

Cílem této práce bylo zjistit, zda lze zlepšit kvalitu prasečích oocytů v *in vitro* kultivačním systému přidáním donoru sulfanu a česnekových derivátů do kultivačního média.

Nejprve byl hodnocen vliv donoru sulfanu na meiotické zrání prasečích oocytů. Bylo zjištěno, že donor sulfanu urychluje jaderné zrání oocytů. Zrychlený průběh jaderného zrání byl doprovázen dřívějším vzestupem aktivity MPF a MAPK. Bylo zjištěno, že akcelerační účinky donoru sulfanu jsou výraznější u oocytů zbavených kumulárních buněk než u celých kumulo-oocytárních komplexů. Dále bylo zjištěno, že donor sulfanu potlačil produkci hyaluronové kyseliny v průběhu meiotického zrání, a že tato inhibice produkce hyaluronové kyseliny je zprostředkována oocytem. Po partenogenetické aktivaci oocytů dozrálých v přítomnosti donoru sulfanu bylo zjištěno, že donor sulfanu zvýšil aktivační potenciál těchto oocytů.

V dalších experimentech byl hodnocen vliv donoru sulfanu na proces stárnutí prasečích oocytů. Bylo zjištěno, že během stárnutí prasečích oocytů dochází k endogenní produkci sulfanu, a že tato endogenní produkce sulfanu klesá po prvních 24 hodinách prodloužené kultivace. Dále bylo zjištěno, že donor sulfanu zcela potlačil fragmentaci prasečích oocytů vystavených prodloužené kultivaci. Inhibitory sulfan uvolňujících enzymů naopak zhoršily kvalitu stárnoucích prasečích oocytů. Donor sulfanu zlepšil také kvalitu stárnoucích prasečích oocytů ošetřených inhibitory sulfan uvolňujících enzymů. Donor sulfanu zcela potlačil fragmentaci oocytů ošetřených současně inhibitory enzymů CBS a CSE a oocytů ošetřených současně inhibitory enzymů CSE a 3-MPST. Donor sulfanu částečně potlačil také fragmentaci oocytů ošetřených současně inhibitory enzymů CBS a 3-MPST a oocytů ošetřených současně inhibitory všech tří sulfan uvolňujících enzymů. Dále bylo zjištěno, že přítomnost donoru sulfanu během prodloužené kultivace oocytů zlepšuje jejich časný embryonální vývoj po partenogenetické aktivaci.

Dále byl hodnocen vliv česnekových derivátů alliinu a S-allyl cysteinu na meiotické zrání prasečích oocytů. Bylo zjištěno, že zatímco alliin narušuje průběh jaderného zrání oocytů, S-allyl cystein nemá vliv na průběh jaderného zrání, aktivitu MPF a MAPK, ani produkci hyaluronové kyseliny během meiotického zrání oocytů. Dále bylo zjištěno, že S-allyl cystein urychluje nástup rýhování embryí a snižuje hladiny ROS ve zrajících a partenogeneticky aktivovaných oocytech.

Na základě výsledků těchto experimentů lze konstatovat, že kvalitu oocytů v *in vitro* kultivačním systému lze zlepšit dodáním donoru sulfanu a česnekového derivátu S-allyl cysteinu do kultivačního média.

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9 Přílohy

Příloha 1: **Vliv alliinu na jaderné zrání oocytů po 20 hodinách kultivace**

	Stádium jaderného zrání (% ± S.E.M.)				
	GV	LD	MI	AITI	MII
Kontrola	29,3±0,8[a]	25,2±1,8[a]	45,4±1,1[a]	0,0±0,0[a]	0,0±0,0[a]
Alliin (0,05 mM)	28,1±0,8[a]	26,6±1,2[a]	45,3±1,4[a]	0,0±0,0[a]	0,0±0,0[a]
Alliin (0,1 mM)	43,4±1,4[b]	24,7±2,2[a]	30,7±1,1[b]	1,2±1,2[a]	0,0±0,0[a]

a, b – statisticky významné rozdíly v dosaženém stádiu meiotického zrání ($p < 0,05$)

Příloha 2: **Vliv alliinu na jaderné zrání oocytů po 30 hodinách kultivace**

	Stádium jaderného zrání (% ± S.E.M.)				
	GV	LD	MI	AITI	MII
Kontrola	0,0±0,0[a]	0,0±0,0[a]	41,3±1,1[a]	54,8±1,9[a]	3,9±1,3[a]
Alliin (0,05 mM)	20,0±2,0[b]	10,0±2,0[b]	28,8±1,3[b]	40,0±2,0[b]	1,3±1,3[a]
Alliin (0,1 mM)	29,1±2,2[b]	10,1±2,0[b]	29,1±1,4[b]	30,5±2,4[c]	1,3±1,3[a]

a, b, c – statisticky významné rozdíly v dosaženém stádiu meiotického zrání ($p < 0,05$)