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

Analytické stanovení hyaluronové kyseliny v kumulo-oocytárním komplexu prasete za účelem hodnocení kumulární expanze

doktorská disertační práce

Autor: **Ing. Kateřina Adámková**

Školitel: **prof. Ing. Jaroslav Petr, DrSc.**

Konzultant: **Ing. Jan Nevoral, Ph.D.**

Praha 2017

Prohlášení

Prohlašuji, že jsem disertační práci na téma: „Analytické stanovení hyaluronové kyseliny v kumulo-oocytárním komplexu prasete za účelem hodnocení kumulární expanze“ vypracovala samostatně a použila jen pramenů, které cituji a uvádím v přiloženém seznamu použité literatury.

V Praze dne:

……

podpis autora

Poděkování

Ráda bych touto cestou poděkovala všem, kteří se podíleli na vzniku mé disertační práce. Především děkuji svému školiteli prof. Ing. Jaroslavu Petrovi, DrSc. nejen za odborné vedení, ale rovněž za jeho vstřícný přístup a velkou ochotu kdykoliv poradit a pomoci. Děkuji svému školiteli specialistovi Ing. Janu Nevoralovi, Ph.D. za nedocenitelnou pomoc při laboratorní práci a za množství cenných odborných i přátelských rad. Ing. Pavlu Kleinovi, Ph.D. děkuji za odborné konzultace a rady při optimalizaci metody. Dále bych ráda poděkovala Ing. Kristýně Hoškové, Ph.D., Ing. Tereze Žalmanové, Ph.D., a Ing. Markétě Dvořákové, Ph.D. za přátelskou i odbornou pomoc. Děkuji také svému manželovi a rodině za velkou podporu během celého studia.

SEZNAM ZKRATEK

3-MPST	3-mercaptopyruvate-sulfurtransferase	3-merkaptopyruvát sulfurtransferáza
AC	Adenylate cyclase	Adenylátcykláza
AI/TI	Anaphase I / Telophase I	Anafáze I / Telofáze I
APC/C	Anaphase promoting complex/cyclosome	Anafázi podporující komplex/cyklosom
BPA	Bisphenol A	Bisfenol A
BPS	Bisphenol S	Bisfenol S
BTH	Hyaluronidase from bovine testes	Bovinní testikulární hyaluronidáza
Ca^{2+}	Calcium ions	Vápenaté ionty
CaMKII	Calmodulin dependent kinase II	Kalmodulin-dependentní proteinkináza II
cAMP	Cyclic adenosine monophosphate	Cyklický adenosin monofosfát
CBS	Cystathionine-beta-synthase	Cystathionin-beta-syntáza
CEEFs	Cumulus expansion-enabling factors	Kumulární expanzi aktivující faktory
cGMP	Cyclic guanosine monophosphate	Cyklický guanosin monofosfát
CO	Carbon monoxide	Oxidu uhelnatý
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
ECM	Extracellular matrix	Extracelulární matrix
EGF	Epidermal growth factor	Epidermální růstový faktor
ERK	Extracellular signal-regulated kinase	Kináza regulovaná extracelulárním signálem
FSH	Follicle-stimulating hormone	Folikulostimulační hormon
FST	Follistatin	Follistatin
GAGs	Glycosaminoglycans	Glykosaminoglykany
GDF-9	Growth differentiation factor-9	Růstový diferenciační faktor-9
GnRH	Gonadotropin releasing hormone	Gonadotropiny uvolňující hormon
GV	Germinal vesicle	Zárodečný váček
GVBD	Germinal vesicle breakdown	Rozpad zárodečného váčku
H_2S	Hydrogen sulfide	Sulfan
HA	Hyaluronic acid	Hyaluronová kyselina
HABPs	Hyaluronic acid-binding proteins	Hyaluronovou kyselinu vázající proteiny
HAS1	Hyaluronan synthase 1	Hyaluronan-syntáza 1

HAS2	Hyaluronan synthase 2	Hyaluronan-syntáza 2
HAS3	Hyaluronan synthase 3	Hyaluronan-syntáza 3
HO	Heme oxygenase	Hem-oxygenáza
HPLC	High-performace liquid chromatography	Vysokoúčinná kapalinová chromatografie
IGF	Insuline-like growth factor	Inzulínu podobný růstový faktor
IL-6	Interleukin-6	Interleukin-6
IP_3	Inositol 1,4,5-triphosphate	Inositol-1,4,5-trifosfát
IP_3R	Inositol 1,4,5-triphosphate receptor	Inositol-1,4,5-trifosfátový receptor
IαI	Inter-α-trypsin inhibitor	Inhibitor inter-α-trypsinu
LD	Late diakinesis	Pozdní diakineze
LH	Luteinizing hormone	Luteinizační hormon
MAPK	Mitogen-activated protein kinases	Mitogeny aktivovaná proteinkináza
MAPKK	Mitogen-activated protein kinase kinase	Kináza mitogeny aktivované proteinkinázy
MAPKKK	Mitogen-activated protein kinase kinase kinase	Kináza kinázy mitogeny aktivované proteinkinázy
MI	Metaphase I	První meiotická metafáze
MII	Metaphase II	Druhá meiotická metafáze
MPF	Metaphase promoting factor	Metafázi podporující faktor
NO	Nitric oxide	Oxidu dusnatý
NOS	Nitric oxide synthase	NO-syntáza
PGCs	Primordial germ cells	Primordiální zárodečné buňky
PGE2	Prostaglandin E2	Prostaglandin E2
PKA	Protein kinase A	Proteinkináza A
RyR	Ryanodine receptor	Ryanodinový receptor
SAC	S-allyl cysteine	S-allyl cystein
SHAP	Serum-derived hyaluronan-associated protein	Od séra odvozený protein asociovaný s HA
SHH	Hyaluronidase from *Streptomyces hyalurolyticus*	Hyaluronadáza ze *Streptomyces hyalurolyticus*
SRY	Sex-determining region on the Y chromosome	Pohlavně determinující faktor chromozomu Y
TGFβ	Transforming growth factor β superfamily	Transformující růstový faktor β
TNFAIP6	Tumour necrosis factor α-induced protein 6	Protein 6 indukovaný faktorem nádorové nekrózy α
uPA	Urokinase-type plasminogen activator	Plazminogenový aktivátor urokinázového typu
Zn	Zincum	Zinek

OBSAH

1 Úvod

Reprodukční biotechnologie představují aplikace poznatků progresivního oboru vývojové biologie. Od úspěšnosti reprodukčních biotechnologií se odvíjí efektivita šlechtění zvířat stejně jako asistované reprodukce humánní medicíny. Limitujícím faktorem jejich rozvoje je zisk dostatečného množství kvalitních vývojově kompetentních oocytů ve stádiu metafáze II. meiotického dělení. Řešení přináší studium meiotického zrání a kumulární expanze. Vhodný model pro studium těchto procesů představuje prasečí kumulo-oocytární komplex, který sdílí řadu podobností s lidským.

Současně s meiotickým zráním oocytu probíhá kumulární expanze kumulo-oocytárního komplexu. Kumulární expanze spočívá v produkci velkého množství extracelulární matrix, zejména hyaluronové kyseliny (HA). Vlivem kumulární expanze dochází postupně k přerušení komunikačních propojení mezi oocytem a kumulárními buňkami, což má za následek izolaci oocytu od regulačních faktorů z kumulárních buněk. Vzhledem k úzkému vztahu kumulární expanze a meiotického zrání oocytu, je kumulární expanzi možné využít jako marker úspěšnosti *in vitro* zrání oocytů stejně jako kvality oocytů dozrálých *in vivo* a vypláchnutých z těla dárkyně pro účely *in vitro* oplození.

Pro hodnocení kumulární expanze byla vyvinuta řada metod. Doposud jsou používané metody vizuálního hodnocení expandovaného kumulu, jež jsou značně nepřesné. Objektivnější metody jsou založeny na měření obsahu HA pomocí imunologické metody ELISA nebo radioaktivního značení prekurzorů syntézy polymeru HA. V současné době je vhodné zaměřit se na vývoj analytických metod, které by stanovily množství HA bez nutnosti využití nákladných protilátek a radioizotopů. Předložená disertační práce mimo jiné popisuje metodu analytického stanovení hyaluronové kyseliny, vyvinutou s respektem k těmto požadavkům. Přesné stanovení HA, poskytující objektivní informace o intenzitě kumulární expanze přináší cenné poznatky o průběhu tohoto procesu a zejména o kvalitě oocytů.

Zavedení a využití metody analytického stanovení HA se uplatní jak v základním vědeckém výzkumu, tak v praktickém použití pro odbornou veřejnost. Ve výzkumu najde uplatnění při testování vlivu látek – pozitivních i polutantů. Je známo, že kumulární expanze citlivě reaguje na přítomnost pozitivních/negativních látek. V disertační práci jsou testovány účinky gasotransmiteru sulfanu, česnekového derivátu S-allyl cysteinu a endokrinního disruptoru bisfenolu S. V praxi pak metoda pomůže kvalifikovaně selektovat oocyty s ohledem na jejich kvalitu pro následné použití v biotechnologických postupech.

2 Literární rešerše

2.1 Oogeneze savců

2.1.1 Fáze množení zárodečných buněk

V průběhu embryonálního vývoje vznikají samičí i samčí gonády ze stejného základu, který je označován jako genitální lišta. Formování genitální lišty probíhá na ventrální části zárodku, kde je patrná jako podlouhlý párový útvar, vznikající z povrchového epitelu somatopleury a mezenchymu nalézajícího se pod ním.

Ve stejné době začínají gonádu osidlovat primordiální zárodečné buňky (PGCs – primordial germ cells), jež slouží jako progenitory samičích i samčích pohlavních buněk. PGCs migrují z kaudální části prvostřeva a žloutkového váčku podél dorzálního mezenteria do povrchové vrstvy genitálních lišt (Sadler 2011). U prasnice se PGCs poprvé objevují okolo 24. dne embryonálního vývoje (Bielanska-Osuchowsky, 2006). Energii k migraci získávají z lipidových kapének a glykogenových partikulí uložených v cytoplazmě (Motta *et al*., 1997). PGCs následně indukují proliferaci epitelu somatopleury, se kterou vytvářejí zárodečný epitel, a tím vznik medulárních provazců v indiferentních gonádách.

Ačkoliv je pohlaví embrya determinováno hned po oplození, v popisovaném stádiu zatím nelze morfologicky určit pohlaví jednice. Pohlaví u savců je determinováno geneticky, kdy absence pohlavně determinujícího faktoru chromozomu Y (SRY – sex-determining region on the Y chromosome) a přítomnost specifických genů jako WNT4 (wingless-type MMTV integration site family member 4), FOXL2 (forkhead box L2) a FST (follistatin), vedou k dalšímu větvení medulárních provazců, vzniku kortikálních pruhů, a tím k přeměně indiferentní gonády ve vaječník (Eicher *et* Washburn, 1986).

Simultánně s tímto procesem PGCs vstupují do mitotického dělení a tvoří se oogonie, které jsou obalovány okolními somatickými buňkami, které v průběhu dalšího vývoje dají vzniknout folikulárním buňkám. Popsaný proces množení PGCs ve velké množství oogonií je označováno jako fáze množení zárodečných buněk. U prasnic je mitotické dělení PGCs zahájeno od 13. dne embryonálního vývoje a trvá až do 7. dne po narození. Počet oogonií přitom nejvýrazněji stoupá mezi 20. a 50. dnem, kdy se jejich počet zvyšuje z 5 000 až na 1 100 000. Po ukončení mitotické aktivity podléhá značná část oogonií atrézii (Hunter, 2000).

Klasické dogma reprodukční biologie předpokládalo vznik oocytů pouze ze zásob namnožených v průběhu embryogeneze z PGCs. Uvedené tvrzení bylo vyvráceno vědeckou skupinou vedenou Jonathanem Tillym (Johnson *et al*, 2004, 2005), jejíž experimenty poukazují na přítomnost zárodečných kmenových buněk v ováriích dospělých myší. Tyto zárodečné kmenové buňky mají svůj původ v kostní dřeni, odkud mohou osidlovat ovárium a vytvářet nové pohlavní buňky (Johnson *et al*., 2005).

Oogonie vstupují již během prenatálního období do meiotického dělení, čímž se z oogonie stává primární oocyt. Charakteristickým znakem dělení oocytu je asymetrické dělení cytoplazmy, čímž vzniká jen jedna funkční gameta a dvě, případně tři pólová tělíska, která se na vzniku zygoty nepodílejí.

Po posledním mitotickém dělení oogonie vstupují do interfáze označované jako *preleptotene*. V průběhu *preleptotene* je dokončena replikace DNA, oogonie jsou obklopovány pre-granulózními buňkami a tenkou bazální membránou. Následně je zahájena profáze prvního meiotického dělení, která zahrnuje pět fází – *leptotene, zygotene, pachytene, diplotene a diakineze* (Wassarman *et* Albertini, 1994).

V průběhu *leptotene* se chromozomy spiralizují a kondenzují, čímž se chromatin mění ve stabilní heterochromatin. V *zygotene* se párují homologní chromozomy a tvoří se bivalenty. V *pachytene* se bivalentní chromozomy zkracují a rozštěpí se na 2 chromatidy. Mezi homologními chromatidami sesterských chromozomů se vyměňují fragmenty DNA, čímž je zajištěna téměř nekonečná variabilita potomstva. Uvedený proces je označován jako crossing-over. V *diplotene* se od sebe začínají jednotlivé chromatidy oddalovat, nicméně doposud zůstávají propojeny v chiazmatech, kde se dokončuje výměna úseků DNA. V posledním stádiu *diakineze* se již chromozomy zcela oddělují a rovněž dochází k prvnímu zastavení meiotického dělení, označovaném jako první meiotický blok (Wassarmann, 1988; Wassarman *et* Albertini, 1994). Primární oocyt má v tomto stádiu dekondenzovaný chromatin a jeho jádro je označováno jako zárodečný váček (GV – germinal vesicle).

2.1.2 Fáze růstu oocytů a folikulogeneze

Pro další vývoj oocytu je důležitá kooperace s buňkami, které ho obklopují. V průběhu fáze růstu oocyt podstupuje řadu morfologických a funkčních změn, z nichž mnohé jsou založené na interakci oocytu a okolních folikulárních buněk. U savců probíhá růst oocytů ve dvou fázích. V rámci první fáze dochází k růstu oocytu v rostoucím folikulu, ve druhé fázi se pak velikost oocytu

nemění a pokračuje pouze folikulární růst (Hunter, 2000). Z hlediska hormonálního řízení rozlišujeme preantrální a antrální fázi folikulogeneze. Průběh preantrální folikulogeneze je nezávislý na působení gonadotropinů, zatímco antrální je již na účinku gonadotropinů závislý.

Na počátku růstové fáze oocyt obklopuje vrstva pre-granulózních buněk a na povrchu tohoto útvaru je vytvořena intaktní bazální lamina. Celý komplex je označován jako primordiální folikul, který představuje základní funkční jednotku ovaria. Teprve v období pohlavní dospělosti dochází na úrovni primordiálních folikulů k růstovým změnám, zahrnujícím zejména intenzivní mitózu a následnou diferenciaci pre-granulózních buněk v granulózní buňky, rovněž označované jako folikulární buňky. V primárním folikulu se folikulární buňky dále mitoticky dělí, čímž vzniká sekundární folikul neboli preantrální folikul, zahrnující dvě a více vrstev folikulárních buněk, jež obklopují oocyt prvního řádu (Wassarman *et* Albertini, 1994; Eppig, 2001). Preantrální fáze folikulogeneze končí v období, kdy se v sekundárním folikulu začne exprimovat receptor pro folikuly stimulující hormon (FSH), čímž se stává vnímavým k působení gonadotropinů (Dierich *et al*., 1998).

Současně s procesem folikulogeneze dochází k růstu oocytu, během kterého oocyt několikanásobně zvětší svůj objem. U prasnice se průměr oocytu zvětšuje z původních 30 μm na 120 - 125 μm, růstová fáze je dokončena ve folikulech o rozměru cca 1,8 mm. Růst oocytu zahrnuje výrazné změny na úrovni uspořádání jádra, morfologie a redistribuce buněčných organel, zvýšenou transkripci, translaci a syntézu nových proteinů, které slouží k regulaci následného meiotického zrání i časného vývoje embrya po oplození.

GV primárního oocytu se v průběhu fáze růstu zvětšuje a mění se jeho uspořádání, čímž se chromatin postupně kondenzuje. Rovněž jadérko roste a postupně mění svojí strukturu z difúzní v kompaktní. Uvedené změny korespondují se zvyšující se syntézou RNA a vysoké transkripci rRNA. Bylo popsáno, že v průběhu fáze růstu se množství RNA v oocytu zvyšuje až 300krát. U plně dorostlých oocytů pak syntéza RNA ustává (Wassarman, 1988).

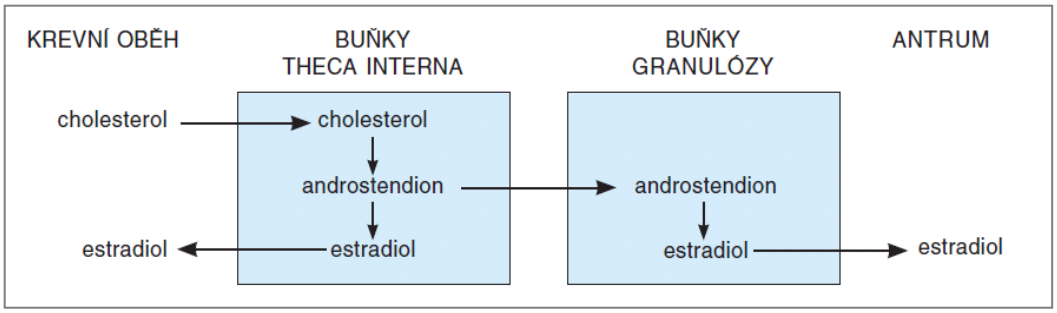
Během růstu dochází k nárůstu počtu mitochondrií a rovněž změně jejich velikosti a morfologie, kdy se z prodlouženého tvaru mění na oválný až kulatý a jejich velikost dosahuje 0,8 μm. Endoplazmatické retikulum se rozšiřuje od GV dále do cytoplazmy (Wassarman, 1988). Strukturální změny postihují rovněž ribozomy a Golgiho aparát, který je v rostoucích oocytech lokalizován na periferii oocytu v blízkosti gap junctions, což souvisí s jejich úlohou při vzniku kortikálních granul. Po ukončení růstu se Golgiho aparát redistribuuje do oblasti GV (Rozinek *et al*., 1991).

Kortikální granula jsou malé organely sférického tvaru ohraničené membránou, které jsou lokalizovány v kortikální oblasti dosud neoplozených oocytů (Wassarman, 1988; Wassarman *et*

Albertini, 1994). Po vniknutí spermie do oocytu dochází ke kortikální reakci, kdy kortikální granula, obsahující proteolytické enzymy a glykoproteiny, fúzují s oolemou a jejich obsah se dostává do perivitelinního prostoru mezi oocytem a *zonou pellucidou*. Uvedeným mechanizmem jsou následně navozeny změny ve struktuře a funkčních vlastnostech *zony pellucidy*, čímž je zabráněno polyspermnímu oplození (Wang *et al.*, 1997).

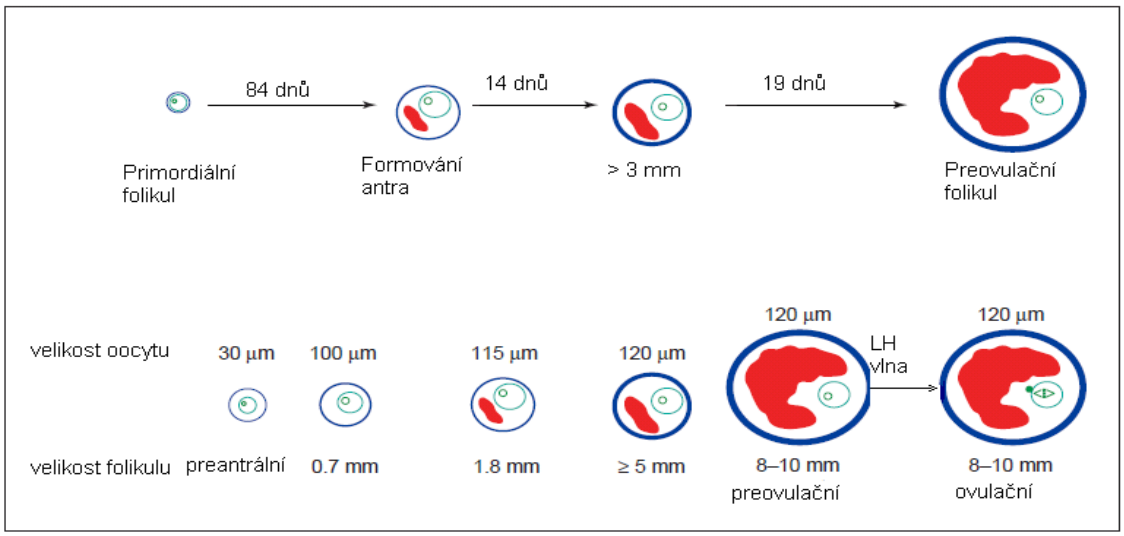
Zona pellucida je glykoproteinový obal oocytu složený z proteinů ZP1, ZP2, ZP3 (zonální protein 1, 2, 3), které tvoří síťovitou strukturu. Spektrum zonálních proteinů se druhově liší, kdy např. člověk má také ZP4. *Zona pellucida* se uplatňuje během oogeneze stejně jako v průběhu oplození, kdy se na její povrchové receptory navazují spermie a spouští akrozomovou reakci.

V průběhu antrální fáze folikulogeneze jsou gonadotropiny pod regulací gonadotropin releasing hormonu (GnRH) uvolňovány z adenohypofýzy. GnRH je produkován hypothalamem, odkud je prostřednictvím hypothalamo-hypofyzárního portálního systému transportován do adenohypofýzy, kde indukuje sekreci gonadotropinů FSH a luteinizačního hormonu (LH). Počáteční nízká sekrece LH aktivuje enzymy v buňkách *theca foliculi interna*, kde indukuje produkci androgenů z cholesterolu. Androgeny jsou následně transportovány do granulózních buněk, kde jsou pod regulací FSH konvertovány enzymem aromatázou na estrogeny (viz. Obr.1).



Obr. 1: Interakce mezi thekálními a granulózními buňkami při syntéze a sekreci estradiolu (převzato Ganong, 2005)

FSH současně indukuje tvorbu receptorů pro LH na granulózních buňkách. Účinkem FSH se rovněž v sekundárním folikulu začíná formovat dutina vyplněná folikulární tekutinou označovaná jako *antrum*, která se v důsledku přibývající tekutiny postupně zvětšuje. Zvyšující se koncentrace estrogenů indukuje zvýšení sekrece LH a tím spouští předovulační LH vlnu, která stimuluje zrání oocytu (Eppig, 2001). Tvorby antrálního folikulu se rovněž účastní apokrinní a parakrinní dráhy, včetně účinku sterioidů a peptidů (Bonnet et al., 2008). Výsledkem je vznik terciálního folikulu neboli preovulačního antrálního folikulu, označovaného také jako Graafův folikul (Eppig, 2001; Hirshfield, 1991). Folikulární vývoj prasečích oocytů je znázorněn na Obr.2.



Obr. 2: Růst prasečích oocytů a folikulů (upraveno dle Hunter, 2000)

Na úrovni Graafova folikulu můžeme folikulární buňky rozdělit na dva základní typy. Kumulární buňky, které v několika vrstvách přímo obklopují oocyt a společně s ním vytvářejí funkční celek označovaný jako kumulo-oocytární koplex (Cumulus oocyte complex – COC), a buňky granulózy. Nejvýznamnějším funkčním rozdílem mezi uvedenými typy buněk je absence receptoru pro LH u kumulárních buněk, jehož exprese je inhibována přítomností oocytu (Eppig *et al.*, 1997). Kumulární buňky se dále diferencují na buňky tvořící vejconosný hrbolek (*cumulus oophorus*) a vrstvu nacházející se v přímém kontaktu s oocytem (*corona radiata*). *Corona radiata* s oocytem komunikuje prostřednictvím buněčných výběžků prostupujících *zonu pellucidu* a mezibuněčných spojů typu gap junction složených z konexinu 37 (Li *et al.*, 2007). Proteinové jednotky gap junction, konexony, jsou v membráně seskupeny do hexagonálního uspořádání a spojují dvě sousední buňky. Vzniklým spojením mohou procházet malé molekuly nepřesahující 1 kDa (Ganong, 2005). Díky tomu jsou do oocytu transportovány malé molekuly z kumulárních buněk – jako cyklický adenosin monofosfát (cAMP) a cyklický guanosin monofosfát (cGMP). cAMP a cGMP mají inhibiční účinky na meiózu (Sun *et al.*, 2009) po celou dobu několikatýdenní fáze růstu oocytu. Dokud rostoucí oocyt nedosáhne konečné velikosti, není znovuzahájení meiotického zrání žádoucí a oocyt setrvává v 1. meiotickém bloku (Moor *et al.*, 1990; Tanghe *et al.*, 2002).

Výsledkem fáze růstu oocytu je plně dorostlý oocyt ve stádiu GV, který dosáhl plné meiotické kompetence. Pouze oocyty s ukončeným růstem jsou plně meioticky kompetentní, schopné dokončit meiotické zrání do metafáze II, kde jsou zastaveny druhým meiotickým blokem.

Plně meioticky kompetentní prasečí oocyty měří 120 – 125 μm a po 48 hodinové *in vitro* kultivaci jsou schopné dosáhnout metafáze druhého meiotického dělení (Motlík *et al*., 1984; Hunter, 2000).

2.1.3 Fáze zrání oocytů

Zrání oocytu představuje přeměnu plně dorostlého meioticky kompetentního oocytu v oocyt schopný oplození. Fáze zrání začíná uvolněním prvního meiotického bloku v primárním oocytu, které je *in vivo* poprvé spuštěno LH vlnou v období puberty. Na LH vlnu mohou reagovat pouze plně meioticky kompetentní oocyty z preovulačního Graafova folikulu. LH spouští signální kaskádu vedoucí k uvolnění prvního bloku meiózy a rozpadu zárodečného váčku (GVBD – germinal vesicle break down), kdy se vlivem rozložení jaderné laminy rozpadá jaderná membrána oocytu (Alberts *et al*., 1998, Hurk *et* Zhao, 2005).

GVBD lze u prasete rozdělit do pěti fází: GV0 – GV4, které se od sebe navzájem liší strukturním uspořádáním chromatinu. Chromatin ve stádiu GV0 je rovnoměrně rozptýlen v celé jaderné oblasti. V GV1 se chromatin začíná postupně kondenzovat a postupně vytváří prstencovitou strukturu, jaderná membrána a jadérko jsou stále neporušeny. V GV2 se tvoří shluky chromatinu v oblasti jaderné membrány. V GV3 se chromatinové shluky rozpadají a pozorujeme filamentární síť. V posledním stádiu GV4 mizí jadérko a jaderná membrána se rozpadá (Motlík *et* Fulka, 1976).

Po GVBD oocyt vstupuje do metafáze I, v průběhu které se se páry homologních chromozomů uspořádají do ekvatoriální roviny dělícího vřeténka. Následují anafáze I a telofáze I, kdy se rozcházejí celé chromozomy. Meióza I přechází do meiózy II bez replikace DNA, buňky nesou poloviční sadu chromozomů. Meióza je přerušena druhým meiotickým blokem v metafázi II, kdy se chromozómy seřadí v ekvatoriální rovině a současně je vyděleno první pólové tělísko. Meiotické dělení je plně dokončeno až po aktivaci oocytu spermií, která způsobí znovuzahájení meiózy. Následuje anafáze II a telofáze II, kdy dochází k vydělení druhého pólového tělíska (Wassarman, 1988).

V průběhu zrání dochází k nezbytným změnám nejen na úrovni jádra, ale také na úrovni cytoplazmy. V průběhu cytoplazmatických změn dochází k redistribuci buněčných organel – mitochondrií, ribozomů, Golgiho aparátu a endoplazmatického retikula prostřednictvím cytoskeletárního aparátu buňky. Rovněž pokračuje syntéza a modifikace molekul potřebných pro zrání i pro období oplození a časný embryonální vývoj (Sirard *et al*., 1993, Ferreira *et al*., 2009).

2.1.4 Regulace meiotického zrání

Corona radiata a cyklické nukleotidy cAMP a cGMP

Důležitou roli ve zrání oocytů mají buňky *corona radiata*, které se společně s buněčnými spoji gap junction podílejí na jaderném zrání oocytu (Sun *et* Nagai, 2003; Karja, 2008). Po hormonálním stimulu LH dochází k morfologickým změnám v COCs, které ve svém důsledku vedou k přerušení gap junction mezi oocytem a kumulárními buňkami, čímž zabraňují transportu cAMP z kumulárních buněk do oocytu (Hurk *et* Zhao, 2005; Liang *et al*., 2007). V oocytu je cAMP snižován také aktivací cAMP-fosfodiesteráz, které cAMP degradují (Mehlmann 2005; Liang *et al*., 2005). Snížení cAMP vede k poklesu protein kinázy A (PKA) a tím k GVBD (Kishimoto, 2003).

Udržení meiotického bloku se rovněž účastní cGMP, který je podobně jako cAMP syntetizován v kumulárních buňkách, odkud je prostřednictvím gap junction transportován do oocytu. cGMP v oocytu inhibuje cAMP-fosfodiesterázu, označovanou také jako PDE3A (Lugnier, 2006), jejíž aktivita je klíčová pro znovuzahájení meiozy u řady živočichů, včetně prasete (Laforest *et al*., 2005; Sasseville *et al*., 2008). Množství cGMP procházející do oocytu z kumulárních buněk je regulováno prostřednictvím LH. Mechanizmy účinku LH na degradaci cGMP byly popsány u myši (Robinson *et al.*, 2012) a jsou obecně založeny na snížení aktivity enzymu syntetizujícího cGMP a snížení propustnosti gap junction. U prasečích oocytů byl po působení LH popsán nárůst degradačního enzymu cGMP (Sasseville *et al*., 2008).

M-fázi podporující faktor

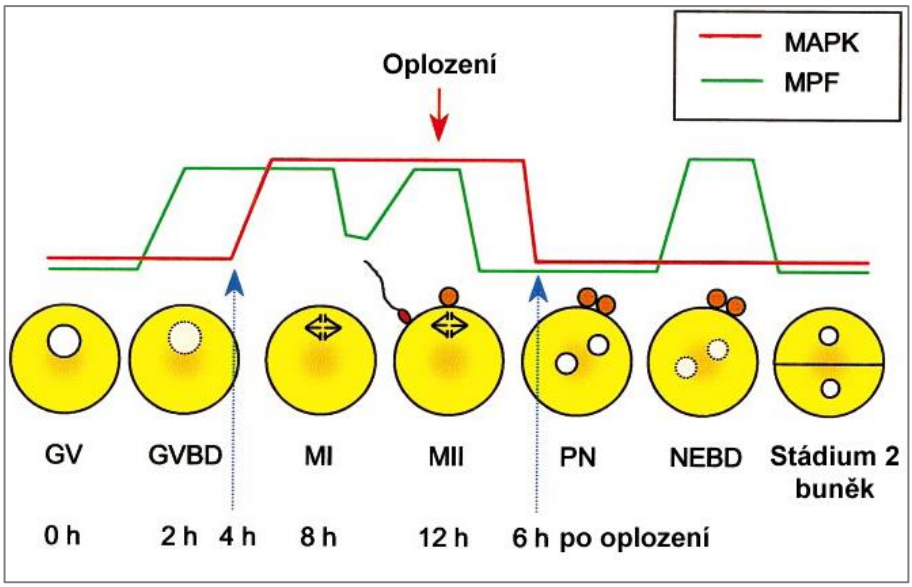
V momentě znovuzahájeného meiotického zrání a GVBD je klíčovou molekulou M-fázi podporující faktor (MPF – M-phase promoting factor). V nezralém oocytu se MPF kumuluje v neaktivní formě – pre-MPF, které sestává z katalytické podjednotky CDK1 a regulační podjednotky cyklinu B. K aktivaci MPF dochází vlivem defosforylace fosfatázou CDC25 a současné fosforylace kinázou WEE1. pre-MPF je udržováno vysokou koncentrací druhého posla cAMP prostřednictvím aktivní PKA. Do momentu GVBD, PKA inaktivuje fosfatázu CDC25 a tak sehrávají cAMP a PKA důležitou úlohu v udržení meiotického bloku (Kishimoto, 2003; Liang *et al*., 2007).

Mitogenem aktivovaná protein kináza

Další důležitou regulační molekulou meiotického zrání je mitogenem aktivovaná protein kináza (MAPK – Mitogen activated protein kinase), označovaná také jako kináza regulovaná extracelulárním signálem (ERK – Extracellular signal-regulating kinase). Na začátku reakční kaskády se nachází molekula proteinu Ras, lokalizovaná uvnitř buňky a nepřímo aktivovaná

prostřednictvím extracelulárního ligandu. Následně dochází k aktivaci kinázy Mos (také MAPKKK – MAPK kinase kinase) a MAPK kinázy (MAPKK), která nakonec aktivuje samotnou MAP kinázu (MAPK) (shrnuto v Liang *et al.*, 2007). V oocytech však není aktivita MAPK striktně závislá na extracelulárních signálech a aktivaci nadřazených aktivačních faktorů Ras a Mos (Fan *et* Sun, 2004). Načasování aktivace MAPK v oocytu je druhově specifické. V případě savčích oocytů je MAPK aktivovaná současně či krátce po GVBD, čímž se stává pro GVBD zbytná (Ye *et al.*, 2003; Hurk *et* Zhao, 2005). Naopak u žab *Xenopus laevis* aktivace MAPK předchází procesu GVBD a je pro jeho aktivaci nepostradatelná (Haccard *et al*., 1995). U savců je MAPK důležitá pro znovuzahájení a správný průběh meiózy, dále reguluje tvorbu dělícího vřeténka a aktivuje MPF prostřednictvím fosforylace proteinu konexinu 43, který se nachází ve struktuře ovariálního gap junction, čímž snižuje průchod výše uvedených inhibičním molekul (Liang *et al.*, 2007). MAPK se rovněž účastní inhibiční fosforylace cdk2, která je připisována molekule Myt1. MAPK v tomto procesu fosforyluje molekulu označovanou jako p90, která následně může vázat C-terminální doménu Myt1, čímž je Myt1 inaktivována (Palmer *et al*., 1998).

Dynamika MAPK a MPF se během meiózy liší (viz Obr. 3). Aktivita MAPK vzrůstá v období kolem GVBD a během přechodu z metafáze I (MI) do metafáze II (MII) zůstává konstantní. Aktivita MPF vrůstá krátce před GVBD a na rozdíl od MAPK dochází k poklesu v období metafáze I a následnému nárůstu aktivity před metafází II (Fan *et* Sun, 2004). Inaktivace MPF v průběhu přechodu z MI do MII probíhá prostřednictvím proteolytické degradace cyklinu B1 katalyzované proteinovým komplexem Anaphase promoting complex/cyclosome (APC/C), který cyklin B1 označí molekulami ubiquitinu, a tím ho determinuje k degradaci v proteasomu – zde označovaný jako cyclosom (Jones, 2004). Po MII fázi buněčného cyklu je hladina MPF i MAPK vysoká. Některé komponenty MAPK-signální kaskády, jako např. Mos, jsou součástí tzv. cytostatického faktoru (CSF), který zapříčiňuje druhý blok meiózy v metafázi II. Komplex CSF zahrnuje především proteinové kinázy, které se následně účastní aktivace signální dráhy MEK-MAPK-p90. K poklesu MPF dochází po oplození, po úplném dokončení meiotického dělení oocytu. V případě MAPK k poklesu dochází v období formování prvojader, tj. v období S-fáze před prvním mitotickým dělením embrya (Fan *et al.*, 2002).



Obr. 3: Aktivita MPF a MAPK během meiotického zrání oocytu (upraveno dle Fan *et* Sun, 2004)

Ionty Ca^{2+} a Zn

Na regulaci zrání se rovněž podílejí ionty vápníku (Ca^{2+}) a zinku (Zn). Ca^{2+} reguluje zrání prostřednictvím změny svojí koncentrace v kumulárních buňkách a posléze také v oocytu. V kumulárních buňkách se koncentrace Ca^{2+} navyšuje po působení předovulační LH vlny. Pomocí gap junction se do oocytu dostávají Ca^{2+} přímo nebo se zde uvolňují z intracelulárních depozit prostřednictvím ryanodinových receptorů (RyR) nebo inositol-1,4,5-trifosfátových receptorů (IP_3R). V případě RyR je ligandem cADPribóza, v případě IP_3R pak inositol-1,4,5-trifosfát (IP3) (Petr *et al*., 2002), který rovněž prochází kanály gap junction z kumulárních buněk (Mattioli et al., 1998). V oocytu se Ca^{2+} stává součástí Ca^{2+}/kalmodulin dependentní proteinkinázy (CaMKII), která inaktivuje adenylátcyklázu (AC), což zapříčiní pokles cAMP (Fan *et al*., 2003, Chen *et al*., 2013). V kumulárních buňkách se Ca^{2+} prostřednictvím aktivace MAPK účastní sekrece estrogenů, progesteronu a parakrinních faktorů regulujících zrání oocytu (Ebeling *et al*., 2011).

Zn se účastní regulace zrání hned na několika úrovních. V období prvního bloku meiózy zabraňuje předčasnému GVBD (Kong *et al*., 2012). Dále je nutný pro dokončení meiotického zrání (Kim *et al*., 2010; Bernhardt *et al*., 2011) a pro dosažení a udržení druhého bloku meiózy prostřednictvím regulace aktivity CSF (Kim *et al*., 2011, Bernhardt *et al*., 2012). Hladina Zn je v oocytu regulována prostřednictvím kumulárních buněk, které snižují Zn v ooctu v období před ovulací (Lisle *et al*., 2013).

Gasotransmitery

Mezi další látky, které mohou ovlivnit meiotické zrání, patří gasotransmitery – jednoduché plynné molekuly s fyziologickým účinkem. Molekuly gasotransmiterů jsou ve fyziologických koncentracích uvolňovány živočišnými buňkami, kde se jako druzí poslové účastní signálních regulačních kaskád. Gasotransmitery jsou schopny volně procházet přes plazmatickou membránu a jejich účinek je tak nezávislý na přítomnosti membránového receptoru. Mezi gasotransmitery řadíme molekuly oxidu dusnatého (NO), oxidu uhelnatého (CO) a sulfanu (H_2S) (Mustafa *et al.*, 2009; Wang, 2002). Uvedené molekuly jsou v rámci signálních buněčných drah propojeny a může tak docházet k jejich vzájemnému ovlivnění (Olson *et al*., 2012).

Oxid dusnatý

NO je v buňkách produkován enzymy NO-syntázami (NOSs), které konvertují L-arginin na citrulin a NO. Dosud byly popsány tři izoformy NOSs – endoteliální (eNOS), neuronová (nNOS) a indukovatelná (iNOS) (Lamas *et al*., 1992). V prasečích oocytech a kumulárních buňkách byly identifikovány všechny výše uvedené izoformy NOSs (Ding *et al*., 2012).

V reprodukčních procesech se NO uplatňuje v regulaci sekrece gonadotropinů. U samců se následně podílí na regulaci produkce testosteronu, spermatogeneze, pohlavního chování či erekce penisu (McCann, 1982; Davidoff *et al*., 1996; Burnett, 2002). U samic se rovněž účastní regulace sekrece steroidních hormonů a tím řízení estrálního cyklu, folikulogeneze, meiotického zrání oocytu a ovulaci (Van Voorhis *et al*., 1995; Jablonka-Sharif *et* Olson, 1998; Jablonka-Shariff *et al*., 1999; Jablonka-Sharif *et* Olson, 2000).

Úloha NO na průběh meiotického zrání oocytů byla studována u myši, prasete a skotu. U myší bylo v případě inhibice NOS nebo jejího genového knock-outu zjištěno zvýšené procento oocytů zastavených v metafázi I a oocytů degenerujících (Jablonka-Sharif *et* Olson 2000). Analogické účinky má absence NOS u oocytů skotu (Schwarz *et al*., 2008) a prasete, kde je NOS nezbytná pro GVBD a dosažení metafáze II (Tao *et al*., 2005). Na zrání prasečích oocytů se významně podílí NOS produkovaná v kumulárních buňkách (Chmelíková *et al*., 2010). Při studiu účinku NO na aktivaci oocytů bylo zjištěno, že NO je schopen aktivovat oocyt prasat a žab *Xenopus laevis* (Petr *et al*., 2005; Ješeta *et al*., 2012; Petr *et al*., 2010).

Pravděpodobným mechanizmem účinku NO v reprodukčních procesech je nitrosylace proteinů, např. ryanodinových receptorů, což vede k otevření iontových kanálů pro Ca^{2+} a tím uvolnění Ca^{2+} do cytoplazmy (Iwakiri, 2011).

Oxid uhelnatý

CO je v buňkách tvořen z molekuly hemu, která je pod účinkem enzymu hem-oxygenázy (HO) degradován na CO a biliverdin. HO se řadí mezi tzv. proteiny tepelného šoku a v buňkách se vyskytuje ve třech izoformách: HO1, HO2 a HO3 (Maines, 1988; McCoubrey *et al*., 1997).

V reprodukčních orgánech byl HO detekován u potkanů ve varlatech a děloze (Trakshel *et al*., 1986; Kreiser *et al*., 2003), a u prasat v granulózních buňkách folikulů (Harada *et al*., 2004). Jeho exprese je nezbytná pro správnou funkci vaječníků, folikulogenezi a ovulaci myších oocytů (Zenclussen *et al*., 2012). Role CO v reprodukčních orgánech a procesech spočívá v regulaci hormonální sekrece, kdy se podílí na řízení produkce steroidních hormonů – estradiolu a progesteronu ve vaječníku (Alexandreanu *et* Lawson, 2003). V průběhu březosti se CO podílí na regulaci nástupu porodu, kdy inhibuje kontrakci hladkosvalových buněk dělohy prostřednictvím regulace cGMP (Bainbridge *et* Smith, 2005; Cella *et al*., 2006). Lze předpokládat, že uvedeným mechanizmem – regulací koncentrace cGMP, CO působí i v kumulárních buňkách a oocytu.

Sulfan

H_2S je pod účinkem enzymů cystathione β-syntáza (CBS), cystathionine γ-lyáza (CSE) a 3-merkaptopyruvát sulfurtransferáza (3-MPST) uvolňován z aminokyseliny L-cysteinu (Wang, 2002; Shibuya *et al*., 2009). Produkce H_2S je regulována prostřednictvím negativní zpětné vazby, kdy H_2S inhibuje aktivitu enzymů zodpovědných za jeho produkci (Wang, 2002).

Přítomnost sulfan uvolňujících enzymů byla prokázána v reprodukční soustavě samců i samic. U samců potkana byl popsán výskyt CBS v Sertoliho a Leydigových buňkách a v zárodečných buňkách, CSE byl detekován v Sertoliho buňkách a nezralých zárodečných buňkách (Sugiura *et al*., 2005). CBS a CSE byly detekovány také v lidské erektilní tkáni (di Villa Bianca *et* al., 2009). Sulfan snižuje relaxaci kavernózního tělíska pyje, čímž reguluje erekci (Ghasemi *et al*., 2012).

V samičí reprodukční soustavě se u potkana CBS a CSE vyskytují v děloze a placentě (Patel *et al*., 2009). U člověka byly uvedené enzymy detekovány ve vejcovodu, kde H_2S indukuje kontrakce vejcovodu a tím posun embrya do dělohy (Ning *et al*., 2014). Naopak v děloze se H_2S u potkana i člověka účastní na snižování kontrakcí svaloviny (Sidhu *et al*., 2001, Hu *et al*., 2011). Na úrovni vaječníků byla exprese CBS detekována v granulózních a kumulárních buňkách, která je nezbytná pro správný vývoj folikulů a zrání oocytů *in vitro* (Liang *et al*., 2006; Liang *et al*., 2007).

Pravděpodobný mechanizmus účinku H_2S spočívá v tzv. S-sulfhydrataci, neboli konverzi –SH skupiny cysteinu na –SSH skupinu. Prostřednictvím sulfhydratace jsou post-translačně modifikovány proteiny a tím je modulována jejich aktivita. Mimo jiné se tímto způsobem H_2S

účastní regulace buněčného cyklu u somatických buněk, kde ovlivňuje cAMP/PKA signální dráhu (Njie-Mbye *et al*., 2012). Lze předpokládat, že podobným mechanizmem H_2S zasahuje rovněž do meiotického zrání oocytu a růstu kumulu.

Hladina gasotransmiteru H_2S v buňkách může být navýšena prostřednictvím účinku česnekových derivátů (Louis *et al*., 2012).

Česnekové deriváty

Česnekové deriváty zasahují do signálních kaskád produkce H_2S a rovněž představují důležitý exogenní donor sulfanu v somatických buňkách (Sun *et al*., 2005; Louis *et al*., 2012). V organizmu byl pozitivní vliv česneku prokázán na řadě míst. Česnek má kardioprotektivní, antiproliferační a neuroprotektivní účinky, stimuluje imunitní systém a snižuje oxidativní stres v buňkách (Banerjee *et al*., 2003; Borrelli *et al*., 2007). Na pozitivních účincích česneku se významně podílí sirná sloučenina gama-glutamyl cystein. Gama-glutamyl cystein může být přeměněn hydrolýzou a následnou oxidací na S-allal cystein sulfoxid neboli allin, nebo může být dlouhodobou extrakcí pod vlivem gama-glutamyl transpeptidázy přeměněn na S-allyl cystein (SAC) (Corzo-Martinez *et al*., 2007).

Allin je působením kaskády reakcí postupně přeměněn na další sirné sloučeniny, které disponují biologickou aktivitou – diallyl sulfid (DAS) a polysulfidy diallyl disulfid (DADS) a diallyl trisulfid (DATS) (Miething, 1988). Bylo popsáno, že DATS má protektivní účinky na kardiomyocyty diabetických potkanů, kde snižuje oxidativní stres a apoptózu buněk. Mechazmius účinku spočívá ve stimulaci enzymu CBS prostřednictvím účinku DATS, čímž dochází ke zvýšení produkce H_2S (Tsai *et al*., 2015). V lidských hepatocytech DATS zvyšuje expresi CBS a CSE a tím produkci H_2S, kde má rovněž antioxidační a antiapoptotické účinky (Chen *et al*., 2016).

Efekt SAC na aktivitu CSE byl popsán u potkaních kardiomyocytů. Po aplikaci SAC potkanům, u kterých byl navozen infarkt, došlo ke snížení mortality a poškození srdce. SAC zde má při regulaci H_2S hned dvě funkce. SAC zvyšuje aktivitu CSE a současně funguje jako substrát CSE, kterým je přeměněn na H_2S (Chuah *et al*., 2007).

Negativní vlivy působící na meiotické zrání

Reprodukce může být ovlivněna řadou látek působících na organizmus z vnějšího prostředí. Vedle látek s toxickým efektem, které působí poškození při zvýšených koncentracích, se v posledních letech diskutují rovněž látky označované jako endokrinní disruptory. Endokrinní disruptory jsou definovány jako exogenní látky, které narušují funkci endogenních hormonů, a to i

ve velice nízkých dávkách (Colborn *et al*., 1993). Vzhledem k tomu, že reprodukce podléhá komplexní endokrinní regulaci, mají endokrinní disruptory na reprodukci významný vliv.

Negativní účinky na reprodukci byly popsány u bisfenolu A (BPA). BPA je součástí většiny plastů, odkud je uvolňován do životního prostředí, včetně vody a potravin (Huang *et* Hang, 2010). Expozice nízkým dávkám BPA vede k defektům v samčím i samičím reprodukčním traktu, ke změnám v mozkové sexuální diferenciaci, komplikacím v průběhu těhotenství a abnormalitám v průběhu meiózy oocytů (Can *et al*., 2005; Hunt *et al*., 2009). Mechanizmus účinku BPA spočívá v jeho schopnosti vázat se na estrogenové receptory a rovněž ovlivňuje další cílové struktury estrogenů (Watson *et al*., 2005). Rozkladem BPA vznikají metabolity, z nichž některé mají rovněž estrogenní účinky, srovnatelné i silnější než BPA (Ben-Jonathan *et* Steimetz, 1998). BPA působí také jako antiandrogen (Krüger *et al*., 2008). BPA ovlivňuje celou osu hypothalamus-hypofýza-gonády. U samic ovlivňuje sekreci GnRH z hypothalamu (Patisauel *et al*., 2006) a funkci hypofýzy (Ramos *et al*., 2003), vaječníků (Hunt *et al*., 2003), dělohy (Markey *et al*., 2005) a prsní žlázy (Vandenberg *et al*., 2007b).

Používání BPA v plastech je postupně omezováno a nahrazeno jinými látkami, nejčastěji bisfenolem S (BPS) (Liao *et al*., 2012). Bylo zjištěno, je BPS rovněž disponuje endokrinně disrupčními účinky a je schopen v organizmu napodobovat účinky estrogenů (Michalowicz *et al*., 2015). Negativní vliv BPS byl prokázán na reprodukci ryb (Ji *et al*., 2013), testikulární tkáň savců (Eladak *et al*., 2015) a na funkci hypofýzy u savců (Vinas *et* Watson, 2013). Lze předpokládat, že BPS rovněž ovlivňuje reprodukci u samic savců a může tak ovlivňovat meiotické zrání oocytů (Žalmanová *et al*., 2015) a kumulární buňky.

2.2 Kumulární expanze

Dozrálý MII oocyt většiny savců je za fyziologických podmínek s okolními kumulárními buňkami ovulován z folikulu do vejcovodu, kde je předurčen k oplození spermií. Kumulární buňky krátce před ovulací prodělávají ve folikulu řadu významných biochemických a strukturálních změn, souhrnně nazývaných jako kumulární expanze, která předchází a reguluje meiotické zrání oocytu. V průběhu kumulární expanze kumulární buňky syntetizují a ukládají složky extracelulární matrix (ECM), čímž dochází k nabývání ECM a tím k zvětšování COC (Dekel *et al*., 1979, Eppig, 1979). Pro syntézu ECM je klíčová kooperace oocytu a kumulárních buněk. Kumulární expanzi je možné pozorovat také v podmínkách *in vitro* v průběhu meiotického zrání oocytu (Eppig, 1979; Dekel *et* Beers, 1980; Salustri *et al*., 1989; Chen *et al*., 1993).

Bylo zjištěno, že intenzita kumulární expanze pozitivně koreluje se zráním oocytu *in vitro* a jeho vývojovou kompetencí (Šutovský *et al*., 1994; Procházka *et al*., 2000; Qian *et al*., 2003; Karja, 2008; Ju *et* Rui, 2012; Auclair *et al*., 2013). Kumulární buňky mají rovněž prostřednictvím regulace glutathionu protektivní účinky před oxidativním stresem (Tatemoto *et al*., 2000), popsán byl také jejich efekt v lipidovém metabolismu oocytu (Auclair *et al*., 2013). Z uvedených důvodů se pro kultivaci oocytů *in vitro* doporučuje vybírat oocyty s neporušeným obalem kumulárních buněk (Karja, 2008). *In vivo* je kumulární expanze klíčová pro úspěšnou ovulaci a zachycení COC nálevkou vejcovodu, správný průchod oocytu vejcovodem a oplození (Russell *et* Salustri, 2006).

V průběhu kumulární expanze postupně dochází k mechanickému přerušení gap junction mezi oocytem a kumulárními buňkami, vlivem nabývání ECM. Výsledkem je izolace oocytu od látek majících inhibiční účinky na meiózu – cAMP a cGMP, produkovaných v kumulárních buňkách (Chen *et al*., 1990). Izolace od inhibičních látek je nezbytná pro znovuzahájení meiózy a GVBD (Šutovský *et al*., 1994). Dalším možným způsobem přerušení gap junction je jejich přímé uzavření prostřednictvím fosforylace, regulované interakcemi komponent ECM a receptoru CD44 (Yokoo *et al*., 2010).

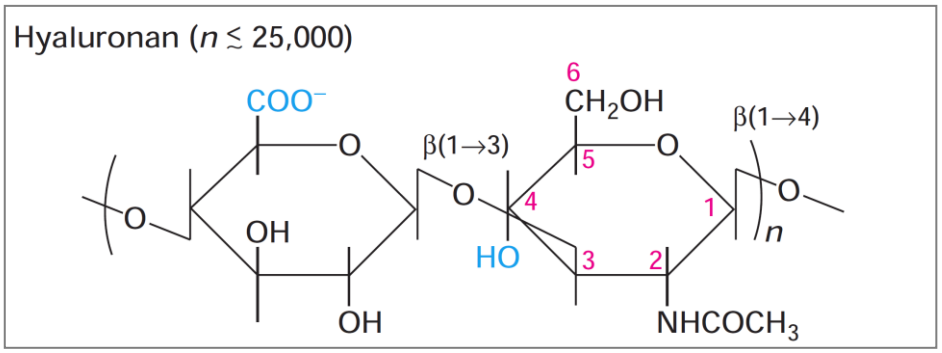
Simultánně s expanzí kumulu dochází k morfologickým změnám kumulárních buněk, které se postupně prodlužují a mění se jejich cytoskelet. K uvedeným změnám dochází v důsledku působení gonadotropinů (Šutovský *et al*., 1994). Morfologické změny se týkají rovněž proteinů gap junction, které jsou endocytovány. To má za následek disagregaci kumulárních buněk v prostoru a zastavení toku inhibičních látek meiózy z kumulárních buněk (Chen *et al*., 1990).

Složení ECM expandovaného kumulu tvoří především glykosaminoglykany (GAGs) a dále proteiny se stabilizační a stavební funkcí pro ECM. Hlavním GAG v COC je hyaluronová kyselina (HA), výrazně méně je zde, oproti jiným tkáním, zastoupen chondroitin sulfát nebo keratin sulfát

(Nakayama *et al.*, 1996; Tirone *et al.*, 1993; Mlynarčíková *et al.*, 2009). Bylo prokázáno, že dostatečný počet vrstev kumulárních buněk spolu s adekvátní produkcí HA v průběhu expanze kumulu jsou klíčové pro úspěšné meiotické zrání oocytu, oplození a časný embryonální vývoj (Chen *et al.*, 1993; Tirone *et al.*, 1993; Kimura *et al.*, 2002; Han *et al.*, 2006; Yokoo *et al.*, 2010). Lze tak předpokládat, že HA je použitelným markerem kvality kumulární expanze a tak i kvality COCs, používaných v postupech reprodukčních biotechnologií (Han *et al.*, 2006).

2.2.1 Hyaluronová kyselina

Hyaluronová kyselina (HA) je tvořena z disacharidových podjednotek β-(1-4)-glukuronové kyseliny a β-(1-3)-N-acetylglukosaminu (viz Obr.4) a řadí se mezi tzv. bezsulfátové GAGs. Disacharidové podjednotky vytvářejí polymery HA, dlouhé od několika opakování až do 25 000. Obvykle má HA polymer okolo 10 000 opakování a celkovou hmotnost 4x 10^6 Da (Lodish *et al.*, 2004). Limitujícím faktorem kumulární expanze je dostatečný počet prekuzrorů synzézy HA (Chen *et al.*, 1990).



β-(1-4)-glukuronová kyselina **β-(1-3)-N-acetylglukosamin**

Obr. 4: Struktura disacharidové podjednotky kyseliny hyaluronové (upraveno dle Lodish *et al.*, 2004)

HA polymer je široce distribuován v ECM pojivové tkáně těla, kde plní řadu rozličných funkcí. Podílí se při opravných procesech při hojení ran (Banerjee *et* Toole, 1992), dále má svou úlohu při přeskupování buněk ve tkáních (Ellis *et al.*, 1997) nebo také při tvorbě metastáz (Zhang *et al.*, 1995). V reprodukci u samic se uplatňuje jako strukturní a signální molekula. Účastní se regulace zrání oocytu (Yokoo *et al.*, 2010), kumulární expanze (Yokoo *et al.*, 2003), ovulace a

oplození (Chen *et al*., 1993), časného vývoje embrya, nidace embrya v děloze (Parikh *et al*., 2006) a morfogeneze plodu (Vabres, 2010).

Syntéza HA je katalyzována membránově vázaným enzymem hyaluronan-syntázou, která se v COCs vyskytuje v několika izoformách. V savčích COCs byly dosud popsány: hyaluronan-syntáza 1 (HAS1), hyaluronan-syntáza 2 (HAS2) a hyaluronan-syntáza 3 (HAS3). U prasnic se vyskytuje HAS2 a HAS3. HAS2 je produkována v kumulárních buňkách, kde se uplatňuje při syntéze lineární molekuly HA během expanze kumulu. Stabilita expandovaného kumulu je zajišťována proteinem označovaným jako TNFAIP6 (tumor necrosis factor-α-induced protein 6) (Rugg *et al*., 2005), a pentraxinem 3, který se rovněž účastní na organizaci ECM (Scarchilli *et al*., 2007). V oocytech prasete byla popsána přítomnost mRNA HAS3 (Kimura *et al*., 2002), HAS3 se zde účastní syntézy HA do perivitelinního prostoru v období zrání oocytu (Ueno *et al*., 2009).

Funkčnost HA závisí na zachování jejího strukturovaného polymeru. Vzhledem k tomu, že není stabilizována propojením s ostatními proteiny ECM ani složkami na bázi sulfátu, využívá tzv. hyaluronovou kyselinu vázající proteiny (HABPs – hyaluronic acid-binding proteins) (Yokoo *et al*., 2002). Jedním z HABPs, který se vyskytuje u COCs prasat je receptor CD44 lokalizovaný v cytoplazmatické membráně kumulárních buněk a v cytoplazmě oocytu. Syntézu receptoru CD44 indukují gonadotropiny v období meiotického zrání oocytu. Bylo popsáno, že interakce CD44-HA jsou důležité pro zrání oocytu v průběhu kumulární expanze (Kimura *et al*., 2002; Yokoo *et al*., 2002; Yokoo *et al*., 2007). Mechanizmus účinku spočívá pravděpodobně ve fosforylaci proteinů gap junction a tím jejich uzavřením pro cAMP (Yokoo *et al*., 2010). Nedostatečné interakce CD44-HA v průběhu *in vitro* kultivace mohou vést k poklesu úspěšnosti meiotického zrání, oplození a časného embryonálního vývoje (Yokoo *et al.*, 2007). Důvodem potlačení vazby HA na receptor CD44 může být glykosylace extracelulární domény CD44 receptoru sialovou kyselinou (Bartolazzi *et al*., 1996).

Protein asociovaný s HA (SHAP – serum-derived hyaluronan-associated protein) je dalším zástupcem HABPs, který se uplatňuje v průběhu kumulární expanze. SHAP stabilizuje a formuje hyaluronovou matrix prostřednictvím kovalentních vazeb s HA (Zhuo *et al*., 2001). SHAP byl u prasat rovněž detekován ve folikulární tekutině a v séru (Nagyová *et al*., 2004). Za formování expandovaného COC prostřednictvím stabilizace HA řetězců je zodpovědný také HABPs TNFAIP6 a inhibitor inter-α-trypsinu (IαI) (Chen *et al*., 1992; Fülöp *et al*., 1997). Stabilizace COC za účasti IαI se děje prostřednictvím ubiquitin-proteasomálního systému, kdy je IαI štěpen na těžké řetězce, které vážou HA, a jsou tak nezbytné pro expanzi COCs u prasat (Nagyová *et al*., 2004).

Degradace HA polymeru je zprostředkována enzymy hyaluronidázami, které jsou lokalizovány v extracelulárním prostředí, kde snižují zastoupení HA v ECM (Lodish *et al*., 2004).

Na základě mechanizmu štěpení HA rozlišujeme tři hlavní skupiny hyaluronidáz. První dvě skupiny jsou endo-β-N-acetyl-hexominidázy. Přičemž první skupina zahrnuje enzymy obratlovců, které substrát hydrolyzují nebo využívají transglykosylační reakce. Druhá skupina zahrnuje převážně enzymy bakteriální – tzv. eliminázy, označované také jako lyázy, které pracují pomocí β-eliminace glykosidické vazby za vzniku nenasycené dvojné vazby. Enzymy třetí skupiny jsou endo-β-glukuronidázy, které polymer štěpí hydrolýzou. Nacházejí se především u pijavic nebo některých korýšů (Stern *et* Jedrzejas, 2006).

2.2.2 Regulace kumulární expanze

In vivo je kumulární expanze řízena gonadotropními hormony FSH a LH. FSH a LH ovlivňují metabolismus granulózních buněk, které vylučují parakrinní faktory mající vliv na kumulární buňky a tím expanzi kumulu. Samotné kumulární buňky nejsou na hormonech FSH a LH přímo závislé (Eppig, 1980; Motlík *et al*., 1998; Zhang *et al*., 2008).

Po působení předovulační LH vlny jsou v granulózních buňkách aktivovány proteázy, které dále aktivují růstový diferenciační faktor 9 (GDF-9 – growth differentiation factor 9). GDF-9 funguje jako parakrinní faktor pro kumulární buňky, kde aktivuje enzymy HAS2 a cyklooxygenázu-2. HAS2 indukuje syntézu HA. Cyklooxygenáza-2 syntetizuje prostaglandin E2 (PGE2), který se po vazbě na příslušné receptory podílí na expanzi kumulu (Richards *et al*., 2002). PGE2 se ve vejcovodu účastní inhibice některých chemokinů, která je důležitá pro remodelaci ECM před oplozením. V případě, že k remodelaci ECM nedojde, stává se rezistentní vůči hyaluronidáze spermie (Tamba *et al*., 2008).

FSH aktivuje buněčnou kaskádu, která se rovněž podílí na stimulaci HAS2 v kumulárních buňkách a tím na syntéze HA (Salustri, 2000). V granulózních buňkách FSH indukuje produkci plazminogenového aktivátoru urokinázového typu (uPA) (Strickland *et* Beers, 1976; Reich *et al*., 1985; Salustri, 2000). Prostřednictvím uPA dochází ke štěpení plazminogenu na aktivní formu proteázy plazmin, který se účastní na uvolnění COC od stěny folikulu během ovulace (Strickland *et* Beers, 1976). uPA inhibují tzv. inhibitory aktivace plazminogenu (Bouton *et al*., 2012).

Předpokládá se, že obě uvedené reakce – aktivace HAS2 a uPA, jsou regulovány stejnou buněčnou kaskádou. Pod účinkem FSH dochází k tvorbě cAMP a k aktivaci PKA. PKA následně aktivuje příslušné transkripční faktory, které na základě faktoru, pocházejícího z oocytu, indukují přepis mRNA HAS2 nebo mRNA uPA (Canipari *et al*., 1995; Salustri, 2000). Uvedeným faktorem

je GDF-9, který spouští přepis mRNA HAS2 a naopak inhibuje přepis mRNA uPA (Elvin *et al*., 1999; Dragovic *et al*, 2005).

Kumulární expanze je regulována rovněž oocytem, prostřednictvím faktorů označovaných jako kumulární expanzi aktivující faktory (CEEFs – Cumulus expansion-enabling factors) (Procházka *et al*., 1998), které jsou produkovány pouze plně dorostlými oocyty v průběhu meiotického zrání (Buccione *et al.,* 1990; Vanderhyden *et al*., 1990; Eppig *et al*., 1993). Po dosažení metafáze I sekrece CEEFs ustává (Nagyová *et al*., 2000). Pro některé živočichy, např. myš, jsou CEEFs pro kumulární expanzi nezbytné. Naopak u prasat expanze probíhá i v případě absence CEEFs (Vanderhyden, 1993; Procházka *et al*., 1998). Pokud se ale z COC odstraní oocyt úplně, dojde k významné redukci syntézy HA a tím i potlačení expanze kumulu, cca o 24 % po 24 hod. kultivace (Nakayama *et al*., 1996; Kimura *et al*., 2002). CEEFs jsou u prasat rovněž produkovány kumulárními a granulózními buňkami a expanze kumulu je tak současně stimulována oocytem a buňkami folikulu (Nagyová *et al*., 1999). Mezi látky identifikované jako součást skupiny CEEFs patří především růstové faktory, zejména tzv. transformující růstové faktory β (TGFβ), kam patří TGFβ1, TGFβ2 a již zmíněný GDF9 (Vanderhyden *et al*., 2003; Dragovic *et al*., 2005).

Expanze kumulu je významným dílem regulována prostřednictvím látek z folikulární tekutiny. Pozitivní vliv byl popsán u epidermálního růstového faktoru (EGF) (Ježová *et al*., 2001) a růstového faktoru podobného inzulinu (IGF-I). Růstové faktory regulují expanzi kumulu prostřednictvím modulace protein-kináz (Němcová *et al*., 2007). Hormony folikulární tekutiny jsou syntetizovány pod účinkem FSH. Bylo zjištěno, že intrafolikulární koncentrace FSH pozitivně koreluje s koncentrací progesteronu a estradiolu ve folikulu (Rosen *et al*., 2009). Ve folikulární tekutině prasete byl rovněž popsán tzv. teplotně-stabilní faktor (heat-stable factor) mající pozitivní účinky na expanzi kumulu (Daen *et al*., 1994). Ve folikulární tekutině jsou rovněž obsaženy faktory s negativním vlivem na meiotické zrání a expanzi kumulu. Mezi látky s inhibičními účinky patří cAMP a PKA, které se účastní inhibičního efektu v plně nedorostlých folikulech (Qian *et al*., 2003).

Dalším parakrinním faktorem regulujícím kumulární expanzi je interleukin-6 (IL-6). IL-6 po vazbě na příslušné receptory aktivuje geny účastnící se expanze kumulu, prostřednictvím klíčových faktorů meiotického zrání, jako je MAPK (Liu *et al*., 2009). IL-6 je modulován tyrosin-kinázovým receptorem A, který je zodpovědný za godadotropně indukovaný vývoj folikulu (Wang *et al*., 2014).

Na regulaci kumulární expanze se rovněž podílejí gasotransmitery. U ovcí byl prokázán účinek gasotransmiteru NO, kdy se přítomnost NOS ukázala jako nezbytná pro adekvátní kumulární expanzi. V případě zablokování NOS docházelo u *in vitro* kultivovaných COCs k potlačení expanze kumulu (Amale *et al*., 2011). Vzhledem k propojení signálních buněčných drah jednotlivých

gasotransmiterů (Olson *et al*., 2012) lze předpokládat, že i další gasotransmitery mohou mít úlohu v regulaci kumulární expanze.

2.2.3 Způsoby hodnocení kumulární expanze

Významu kumulární expanze je již po dlouhou dobu využíváno pro hodnocení kvality COCs. Existuje tak několik způsobů hodnocení kumulární expanze, které lze v zásadě rozdělit do dvou hlavních skupin: 1) první skupina zahrnuje metody založené na vizuálním hodnocení COCs, 2) druhá skupina se soustředí na stanovení obsahu GAGs.

Vizuální hodnocení COCs

Vanderhyden *et al*. (1990) kumulární expanzi hodnotí na základě zařazení COC do jedné z 5 základních skupin podle její intenzity (stupeň 0 až +4).

a) stupeň 0 – COC bez pozorovatelné expanze;
b) stupeň +1 – COC s minimální pozorovatelnou expanzí;
c) stupeň +2 – COC s expanzí v několika vrstvách;
d) stupeň +3 – COC s kompletní expanzí kromě vrstvy *corona radiata*;
e) stupeň +4 – COC s kompletní expanzí zahrnující i vrstvu *corona radiata*.

Obdobný systém využívá také Tao *et al*. (2005), který rozlišuje 3 základní skupiny, které jsou defnovány následovně:

a) 1. skupina: COC s kompletní expanzí ve všech vrstvách kumulárních buněk;
b) 2. skupina: COC s částečnou expanzí (zejména vnějších vrstev kumulárních buněk);
c) 3. skupina: COC bez kumulární expanze.

Odlišnou vizuální metodu popisuje Daen *et al*. (1994), pomocí které stanovuje plochu expandovaného kumulu metodou výpočtu, podle vzorce: plocha [mm^2] = délka x šířka x 0,7854. Plocha COC se měří na předem získaných snímcích COCs, přičemž za délku dosazujeme vzdálenost mezi dvěma nejvzdálenějšími body COC v mm, za šířku pak vzdálenost mezi dvěma nejbližšími body v COC (Daen *et al*., 1994).

Výhodou výše uvedených metod je neinvazivní pracování COCs a možný sběr dat během kultivace *in vitro*. Nevýhoda pak spočívá především v subjektivním hodnocení COCs, ať už přímým zařazením do skupin nebo výběru bodů pro měření plochy kumulu. Z tohoto důvodu je použitelným alternativním způsobem výpočet plochy COC pomocí softwarové analýzy obrazu.

Tato metoda je objektivnější než předchozí zmíněné, ale přesto není prostá společné nevýhody postupů první skupiny, kterou je neschopnost postihnout trojrozměrnou strukturu expandovaného COC.

Měření obsahu GAGs

Metody založené na měření obsahu GAGs využívají biologického poznatku, že v průběhu expanze kumulu dochází k přibývání obsahu GAGs v COC. Nejvhodnějším GAG je HA, jejíž obsah je ve srovnání s ostatními složkami COC nejvyšší.

Doposud často využívanou metodou stanovení obsahu HA je metoda radioaktivního označení prekurzorů syntézy HA (Eppig, 1980; Fagbohun *et* Downs, 1990; Daen *et al*., 1994; Nagyova *et al*., 1999), při které se používá [^{3}H]glukosamin (100 μCi/ml). Prekurzory syntézy jsou aplikovány přímo do kultivačního média, ve kterém probíhá *in vitro* zrání. Po ukončení kultivace následuje extrakce ECM z COC a kultivačního média. Získané vzorky jsou posléze podrobeny enzymatickému štěpení za využití hyaluronidázy (Salustri *et al*., 1990). Obsah HA je stanoven na základě odečtu radioaktivního signálu, který je emitován prekurzory HA (Solursh, 1976). Nevýhodou této metody je práce s radioaktivním materiálem, která je poměrně nákladná a technicky méně dostupná, a vysoký počet COCs ve vzorku.

Mezi další postupy stanovení obsahu HA patří imunologická metoda ELISA (Kongtawelert *et* Ghosh, 1990), spektrofotometrické měření koncentrace štěpných produktů HA (Chen *et al*., 2005) nebo kapalinová chromatografie (HPLC – high-performace liquid chromatography) (Volpi, 2000). Uvedené ne-izotopové metody měření GAGs nejsou doposud rutinně využívány pro hodnocení kumulární expanze COCs, přestože jsou analytické metody ve srovnání s metodami vizuálními významně spolehlivější a přesnější. Jejich nevýhoda spočívá v nemožnosti opakovaného provedení měření na stejném souboru COCs, která vyplývá z nutnosti izolace HA z ECM, jelikož HA polymery jsou navázány v cytoplazmatické membráně buněk (Yokoo *et al*., 2002). Tato nevýhoda je však kompenzována skutečností, že analýze lze podrobit buňky, které se nadále nepoužívají pro další postupy, jako *in vitro* oplození a produkce embryí.

V současné době se tak zdá být nejvhodnější metoda analytického stanovení produktů β-eliminace pomocí spektrofotometrie. Tento postup se zdá být dostatečně přesný a současně ekonomicky příznivý tak, aby našel uplatnění v rutinním využití postupů asistované reprodukce i v základním biologickém výzkumu – např. pro testování účinku látek ovlivňujících meiotické zrání oocytů (např. H_2S) nebo odhadu vlivu polutantů (např. BPS) na zrání oocytů *in vitro*.

3 Hypotéza

Byla stanovena hypotéza, že analytické stanovení hyaluronové kyseliny pomocí měření produktů β-eliminace v *in vitro* kultivovaných COCs může sloužit jako marker kumulární expanze, odpovídající stádiu meiotického zrání oocytu.

Pro potvrzení formulované hypotézy byly stanoveny následující cíle:

1) Zavést metodu analytického stanovení HA v COCs prasete.

   Dílčí cíle:

   - Otestovat dostupné enzymy s hyaluronidázovou aktivitou a optimalizovat metodu spektrofotometrie.
   - Ověřit detekci produktů β-eliminace.
   - Ověřit obsah HA v závislosti na množství COCs.
   - Porovnat metodu spektrofotometrie s vizuálními metodami hodnocení kumulární expanze.

2) Otestovat vliv vybraných faktorů na kumulární expanzi prasečích COCs *in vitro* metodou spektrofotometrie.

   Dílčí cíle:

   - Otestovat vliv gasotransmiteru sulfanu na kumulární expanzi COCs prasete *in vitro*.
   - Otestovat vliv česnekového derivátu SAC na kumulární expanzi COCs prasete *in vitro*.
   - Otestovat vliv endokrinního disruptoru BPS na kumulární expanzi COCs prasete *in vitro*.

4 Materiál a metodika

4.1 Použité chemikálie

Veškeré chemikálie pro analytické stanovení HA byly pořízeny od firmy Sigma-Aldrich Co. (St. Louis, USA), pokud není uvedeno jinak.

- Fosfátový pufr, pH 7,4 (PBS, P5368)
- Polyvinyl alkohol (PVA, 341584)
- Hyaluronan (49775)
- Hyaluronadáza ze *Streptomyces hyalurolyticus* (SHH, H1136)
- Bovinní testikulární hyaluronidáza (BTH, H3506)
- Proteáza z *Bacillus licheniformis* (P4860)

4.2 Měření polymeru HA a standardů oligomerů HA pomocí spektrofotometrie a HPLC

Ke stanovení obsahu HA byla provedena enzymatická digesce HA polymeru prostřednictvím β–eliminace glykosidické vazby specifickou hyaluronidázou ze *Streptomyces hyalurolyticus* (SHH) (Vanderhyden, 1993). Při této chemické reakci je HA polymer enzymaticky štěpen na dimery (HA2) a další oligomery HA obsahující ve své struktuře dvojné vazby. Současně byly analyzovány standardy HA oligomerů (Contipro Group s.r.o., Czech Republic): tetramery (HA4), hexamery (HA6), oktamery (HA8) a dekamery (HA10). Oligomery HA byly detekovány spektrofotometrickým proměřením v ultrafialovém (UV) absorpčním spektru. Vzorky byly spektrofotometricky měřeny v kyvetách Einmal-Küvetten, UV-Küvette mikro (7592 00, Plastibrand) na přístroji Helios Gamma (Spectronic, Thermo Fisher Sci) při vlnové délce 216 nm proti blanku, který sestával z příslušného média a enzymu, bez obsahu HA.

Současně byla provedena analýza pomocí HPLC. HPLC analýza byla provedena na přístroji UltiMate 3000 (Thermo, USA), vybaveném automatickým odběračem vzorků (10 °C), kolonou (35 °C) a PDA detektorem (nastavený při vlnových délkách 210 a 235 nm). Objem vstřikování 25 µl, průtok mobilní faze 1,5 ml/min, celková doba chodu 40 min. Separace analytu byla provedena na Shodexové koloně (IEC QA-825) za využití gradientu eluce. Mobilní faze sestávala z A) 0,02 M roztoku sodium chloridu a vody a B) 0,25 M roztoku sodium chloridu a vody. Gradient

byl následující: 0 min – 100 % A, 25 min 24 % A, 26-32 min – 100 % B, re-ekvilibrace 33-40 min 100 % A. V obou případech byla použita polynomická osmi-bodová kalibrační křivka (od 7 µl/ml do 1000 µl/ml).

4.3 *In vitro* kultivace COCs a hodnocení meiotického zrání oocytu

Prasečí vaječníky byly získávány od necyklujících prasniček poražených na jatkách (Jatky Plzeň a.s., Plzeň, Česká republika), během transportu do laboratoře byly uchovávány při teplotě 39 °C. COCs byly odebírány z ovariálních folikulů o rozměru 2 – 5 mm aspirací 20-G jehlou. Do dalších experimentů byly použity pouze plně dorostlé oocyty s intaktní cytoplazmou, oblopené kompaktním kumulem. Zrání COCs probíhalo v modifikovaném mediu M199 (Sigma-Aldrich), obohaceném o 32,5 mM sodium bikarbonát; 2,75 mM kalcium L-laktát; 0,025 mg/ml gentamicin; 6,3 mM HEPES; 13,5 iu eCG: 6,6 iu hCG/ml (P.G.600; Intervet International B.V., Boxmeer, Holandsko) a 5% (v/v) fetální bovinní sérum (Sigma-Aldrich). Kultivace COCs probíhala 0 – 48 hod. v 4-jamkových Petriho miskách (Nunc, Thermo Fisher Scientific, Inc., Waltham, MA, USA) obsahujících 1,0 ml kultivační média, při 39 °C ve směsi 5,0% CO_2 ve vzduchu.

Experimentální skupiny byly ošetřeny inhibitory sulfan uvolňujících enzymů, česnekovým derivátem SAC a endokrinním disruptorem BPS. Jako inhibitory sulfan uvolňujících enzymů byly použity v trojkombinaci (3Ci) kyselina oxamová, DL-propargylglycin a kyselina α-ketoglutarová, inhibující specificky CBS, CTH a 3-MPST. V experimentech byla použita efektivní koncentrace trojkombinace inhibitorů – 2,0 mM kyselina oxamová, 2,0 mM DL-propargylglycin a 5,0 mM kyselina α-ketoglutarová. Použitý česnekový derivát SAC byl testován při koncentracích 0,1 mM; 0,5 mM a 1,0 mM. Endokrinní disruptor BPS byl testován při koncentracích 3,0 nM; 300 nM a 30 µM.

Po ukončení kultivace byly oocyty zbaveny kumulárních buněk pomocí opakovaného pipetování, montovány na mikroskopická sklíčka s vazelínou, překrytá krycím sklíčkem, a fixována v ethanolu a kyselině octové (3:1, v/v). Oocyty byly obarveny 1,0% roztokem orceinu v 50% vodném roztoku kyseliny octové a jejich stádium meiotického zrání bylo vyhodnoceno pod mikroskopem s fázovým kontrastem. Podle kritérií publikovaných Motlíkem a Fulkou (1976) bylo stanoveno 5 kategorií jaderného zrání: GV – zárodečný váček, LD – pozdní diakineze, MI – metafáze I, AITI – přechod anafáze I do telofáze I, MII – metafáze II.

4.4 Klasifikace expandovaného kumulu

Kumulární expanze byla vizuálně hodnocena pomocí subjektivního 5 – stupňového systému podle Vanderhyden *et al*. (1990). Stupně 0 až +4 korespondují se zvyšující se intenzitou expanze. Stupeň 0 indikuje stádium bez pozorovatelné expanze, +1 indikuje minimální pozorovatelnou expanzi, +2 expanzi rozšířenou v několika vrstvách, +3 kompletní expanzi kromě vrstvy *corona radiata,* a +4 komletní expanzi zahrnující vrstvu *corona radiata*. Data byla vyjádřena jako procentuální část z populace COCs.

4.5 Měření plochy COCs

Pro měření plochy expandovaného kumulu byl použit postup publikovaný Daen *et al*. (1994) s provedenými modifikacemi. Za využití monochromatické CCD kamery (ProgRea CT1, Jenoptik, Německo) se softwarem NIS Elements (Laboratory Imaging, Česká republika) byly každých 8 hod. zhotoveny snímky stejných skupin COCs (25x COCs/skupina). Následně byla provedena analýza obrazu za využití prahovaní a měření plochy 25 COCs. Data byla vyjádřena relativně k COCs po 48 hodinové kultivaci.

4.6 Zpracování expandovaného kumulu a izolace HA

Skupiny po 25 COCs byly kultivovány v 1 ml kultivačního média M199, za výše uvedených podmínek, v časovém intervalu od 0 do 48 hod. Po ukončení kultivace byly COCs 4x propláchnuty v 500 µl PBS-PVA a oocyty byly odděleny mechanicky opakovaným pipetováním v průběhu posledního proplachu. Ke stanovení zadržené HA v expandovaném kumulu byly expandované kumuly přeneseny do mikrozkumavky v 500 µl PBS-PVA a následně enzymaticky štěpeny za využití hyaluronidázy ze *Streptomyces hyalurolyticus* (SHH, 2 iu/ml, Sigma), při 39 °C přes noc. Vzorky zadržené HA v COCs byly uchovávány při -20 °C do proměření. Po spektrofotometrické analýze byla syntéza HA vyjádřena jako koncentrace HA (µg/ml) v získaném roztoku.

4.7 Statistická analýza

Všechny experimenty byly zopakovány nejméně třikrát. Data byla analyzována postupem obecných lineárních modelů (GLM – General Linear Models) v softwaru SAS (Statistical Analysis Systém, Version 9.3, 2012). Významné rozdíly mezi skupinami byly stanoveny pomocí t-testu a Sheffeho testu. Hladina významnosti byla stanovena na $P < 0,05$.

4.8 Design experimentu

Testování enzymů s hyaluronidázovou aktivitou a spektrofotometrická optimalizace

Cílem experimentu bylo vyhodnotit nevhodnější kombinaci enzymu, štěpícího polymer HA, a inkubačního média. Byly použity dva enzymy tvořící heterodimery a oligomery z HA polymeru, které se odlišují mechanizmem digesce HA: a) lyáza SHH s β-eliminázovou aktivitou jakožto enzym specificky degradující HA (Vanderhyden 1993), s tvorbou nenasycených vazeb absorbujících světlo v UV absorpčním spektru při 232 nm (Yosizawa et al., 1983); b) bovinní testikulární hyaluronidáza (BTH), hydrolytický enzym tvořící nasycené vazby, které v UV spektru světlo neabsorbují (negativní kontrola). Přítomnost produktů degradace HA byla prokázána metodou HPLC. Současně byla provedena optimalizace spektrofotometrie zaměřená na nalezení vlnové délky s maximálním výnosem absorbance.

Ověření detekce produktů β-eliminace

Cílem experimentu bylo prokázat absorbanci stadardů HA oligomerů (HA4 – HA10) bez digesce enzymem. Paralelně bylo provedeno měření HA polymeru s využitím a bez využití SHH (SHH+ a SHH-) pro digesci za účelem ověření specifity měření produktů β-eliminace. Absorbance byla měřena spektrofotometricky při 216 nm.

Ověření obsahu HA v závislosti na množství COCs

Cílem experimentu bylo ověřit závislost obsahu HA na počtu COCs ve vzorku a prokázat tak specifitu HA analýzy. Testovány byly vzorky po 15-ti, 25-ti a 50-ti COCs, připravené po 48 hod. kultivace *in vitro*. HA byla spektrofotometricky analyzována při výše uvedené optimalizované vlnové délce. Současně byla provedena kontrola jaderného zrání oocytů.

Porovnání jednotlivých metod hodnocení kumulární expanze

Cílem experimentu bylo porovnat výsledky vizuálních a analytických metod hodnocení kumulární expanze. Testovány byly 3 metody stanovení expanze kumulu: vizuální hodnocení COCs (Vanderhyden, 1990), měření plochy COCs (Daen *et al.*, 1994) a spektrofotometrická analýza HA, na stejné populaci COCs komplexů (25 COCs/skupina). Pro tento experiment byly COCs testovány každých 8 hod. po dobu 48 hod. Současně byly shromážděny výsledky meiotického zrání oocytů.

Testování vybraných faktorů na průběh kumulární expanze *in vitro*

Cílem experimentu bylo vyhodnotit vliv vybraných faktorů na kumulární expanzi *in vitro*. Do vybraných faktorů byly zařazeny – gasotransmiter sulfan, česnekový derivát SAC a endokrinní disruptor BPS. Analýza probíhala za využití spektrofotometrické metody. Současně byla provedena kontrola jaderného zrání oocytů.

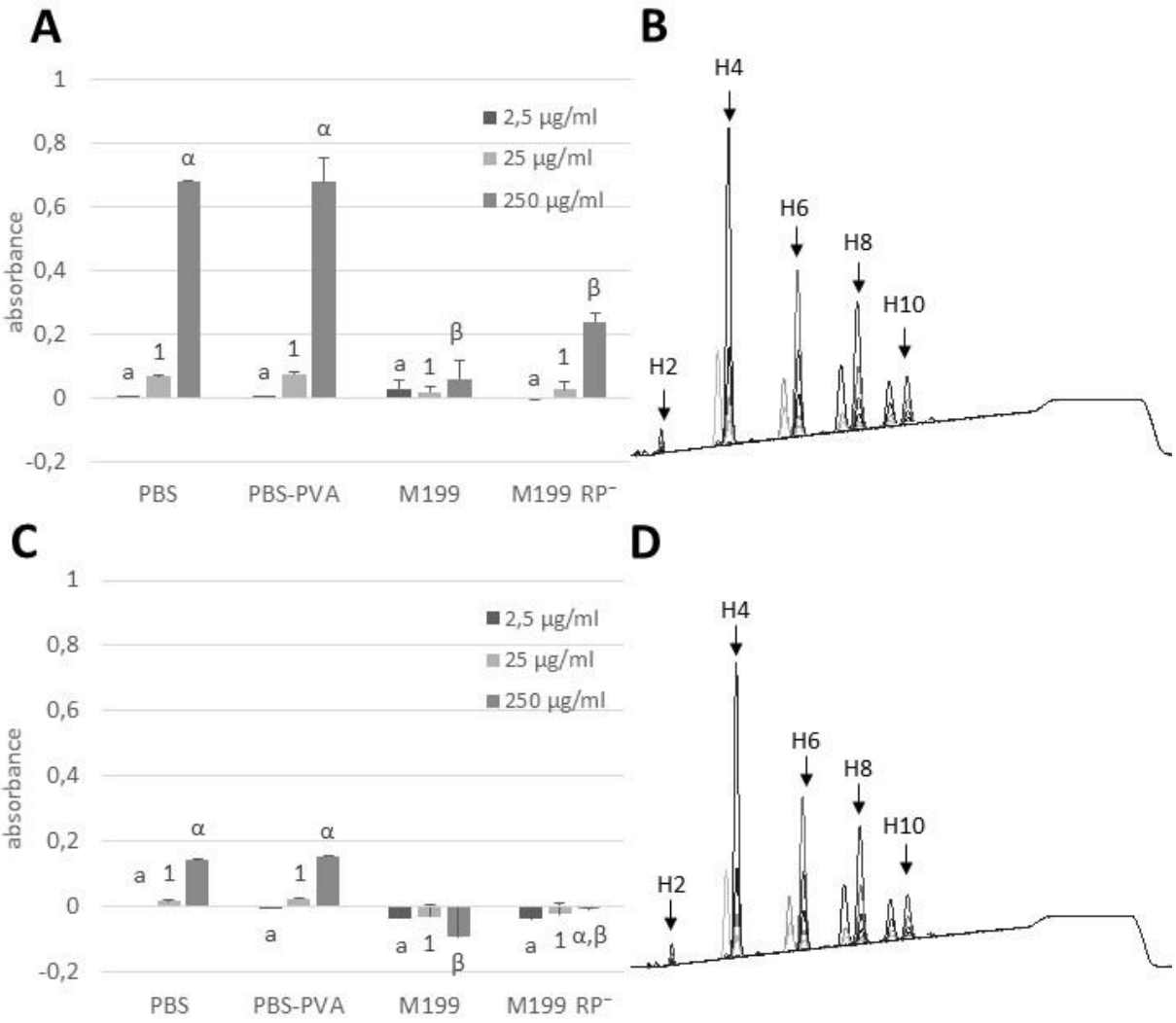
5 Výsledky

5.1 Zavedení analytické metody spektrofotometrie a její porovnání s metodami vizuálními.

5.1.1 Testování enzymů s hyaluronidázovou aktivitou v odlišných médiích a optimalizace vlnové délky pro spektrofotometrické měření.

Cílem experimentu bylo určit optimální médium pro digesci enzymu lyázy ze *Streptomyces hyalurolyticus* (SHH) a optimální vlnovou délku pro spektrofotometrickou analýzu s maximálním výnosem absorbance. Bylo zjištěno, že optimální kombinaci představuje SHH v PBS. Srovnatelné výsledky poskytuje i kombinace SHH v PBS-PVA (0,01%), kde přídavek PVA je nezbytný pro manipulaci s COCs. Média M199 a M199 bez fenolové červeně (M199 RP^-), běžně používaná pro kultivaci COCs, nejsou pro digesci SHH a spektrofotometrickou analýzu vhodná, hodnoty absorbance byly signifikantně nižší (Obr. 1A). Současně byla provedena negativní kontrola za použití bovinní testikulární hyaluronidázy (BTH), kdy jsme předpokládali absenci produktů β−eliminace. V souladu s tímto předpokladem nebyla při spektrofotometrickém proměření absorbance detekována (Obr. 1C). Detekce HA dimerů a oligomerů byla provedena pomocí HPLC (Obr. 1B a 1D). Pro spektrofotometrické stanovení byl a použita vlnová délka 232 nm v UV spektru, v souladu s předchozím doporučením (P. Klein, pers. comm.).

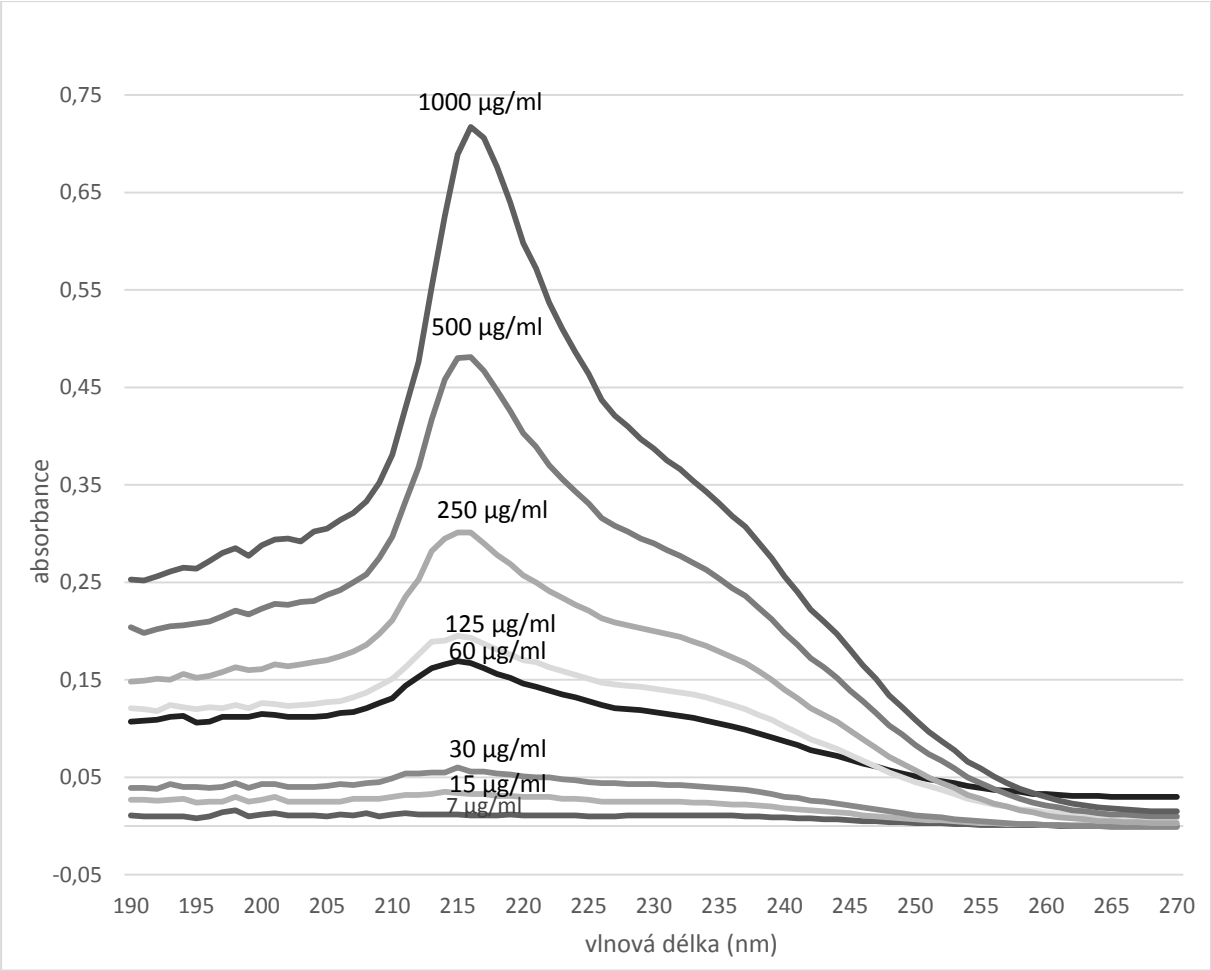
Následně byla kombinace SHH-PBS použita pro přípravu standardů, které byly proměřeny v rozmezí 190-280 nm pro zjištění optimální vlnové délky. Nejvyšší hodnoty absorbance byly naměřeny při 216 nm u všech použitých standardů HA (Obr. 2), uvedená vlnová délka byla použita v následujících experimentech. Současně byl stanoven detekční limit spektrofotometrie, absorbance rovna ~0.13, kdy 60 μg/ml HA polymeru byla určena jako minimální koncentrace pro detekci produktů β−eliminace (Obr. 2).



Obrázek 1) Testování optimální kombinace enzymu s hyaluronidázovou aktivitou a média.

Přítomnost produktů digesce hyaluronové kyseliny (HA) účinkem lyázy ze Streptomyces hyalurolyticus *(SHH) (A, B) a bovinní testikulární hyaluronidázy (BTH) (C, D). Porovnání odlišných kombinací enzym-médium pro HA digesci a spektrofotometrické měření (vlnová délka 232 nm). Použity byly SHH s β–eliminázovou aktivitou (A) a BTH s hydrolytickou aktivitou (C). Data byla ověřena za použití HPLC (210 nm a 235 nm vlnové délky pro detekci nenasycených a nasycených vazeb, ředěno v PBS), výstupy jsou uvedeny na příslušných chromatogramech (B, D). Sloupce ukazují průměr 3 nezávislých experimentů ± SEM;* $^{a,A;1,I;\alpha,\beta}$*odlišné superskripty značí statisticky významné rozdíly (P < 0,05).*

PBS = fosfátový pufr, PBS-PVA = 0,01% polyvinyl alkohol ve fosfátovém pufru, M199 = kultivační médium M199, M199 RP⁻ = médium M199 bez fenolové červeně.



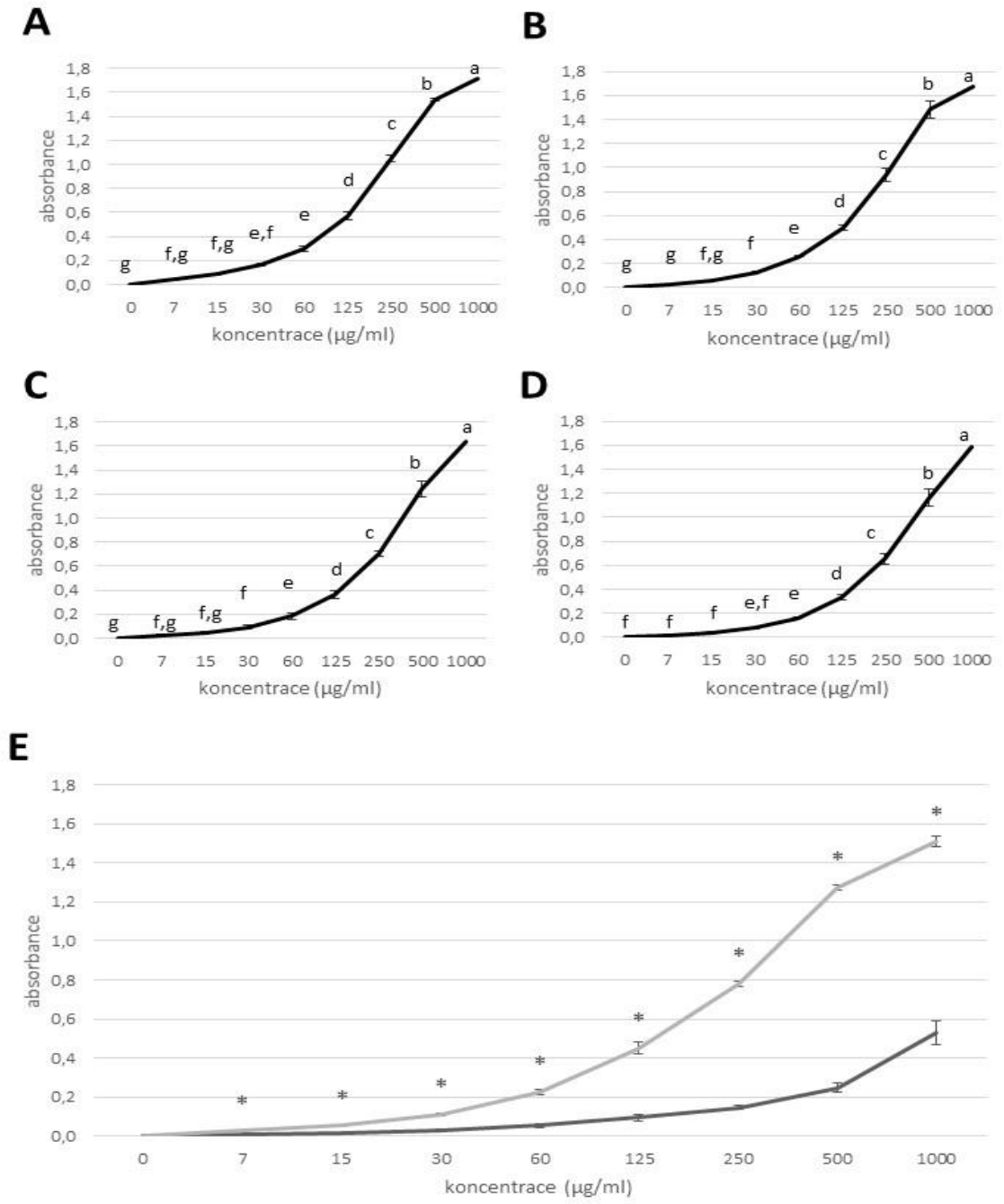
Obrázek 2) Optimalizace vlnové délky pro spektrofotometrické měření.

Optimalizace vlnové délky byla provedena za použití roztoků HA standardů a koncentraci 7–1000 µg/ml, které byly štěpeny SHH v PBS. Produkty β–eliminace byly proměřeny při vlnových délkách v rozmezí 190 – 270 nm.

HA = hyaluronová kyselina, SHH = lyáza ze Streptomyces hyalurolyticus, *PBS = fosfátový pufr.*

5.1.2 Měření standardů roztoku HA polymeru a jeho oligomerů

Pro ověření, že je při spektrofotometrické analýze detekován štěpený HA polymer, byly použity oligomery HA (4HA – 10HA, obsahující 2-5 heterodimerů kyseliny β-(1-4)-glukuronové a β-(1-3)-N-acetylglukosaminu). Získaná data potvrzují předešlá měření polymeru HA po digesci SHH jako výsledek přítomnosti produktů β−eliminace se srovnatelnou absorbancí (Obr. 3). V případě neštěpeného HA polymeru byla absorbance rovněž detekována, její hodnota byla ale signifikantně nižší, přibližně 500 μg/ml neštěpené HA odpovídá hodnotě absorbance 60 μg/ml HA po digesci SHH (Obr. 3E). Limity detekce standardů oligomerů 4HA – 10HA jsou odlišné v závislosti na délce oligomeru. Pro oligomery 4HA - 6HA byl stanoven limit detekce 30 μg/ml HA. Detekční limit pro oligomery 8HA – 10HA je srovnatelný s detekčním limitem produktů β−eliminace, tj. 60 μg/ml HA (Obr. 3A – D).

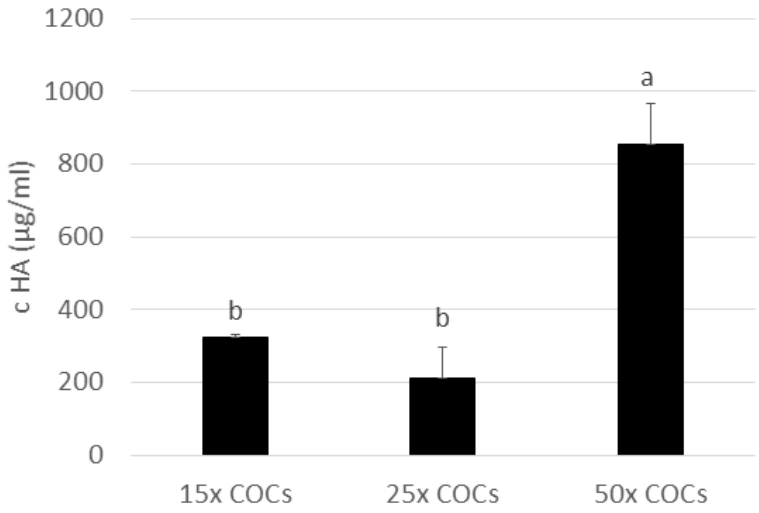


Obrázek 3) Měření standardů oligomerů a polymeru hyaluronové kyseliny.

Měření standardů neštěpených oligomerů HA – 4HA (A), 6HA (B), 8HA (C) a 10HA (D). Naměřena byla bazální hladina absorbance neštěpeného HA polymeru (SHH$^-$) a porovnána s HA polymerem po digesci SHH (SHH$^+$) (E). Standardní roztoky byly rozředěny v PBS a spektrofotometricky proměřeny při vlnové délce 216 nm. Body ukazují průměry 3 nezávislých experimentů ± SEM; $^{a,b,c,d,e,f;*}$*odlišné superskripty značí statisticky významné rozdíly (P < 0,05). SHH = hyaluronan lyáza ze* Streptomyces hyalurolyticus*, PBS = fosfátový pufr*

5.1.3 Závislost množství HA na počtu COCs ve vzorku

Cílem experimentu bylo prokázat, že obsah HA je aplikovatelným markerem pro hodnocení kumulární expanze COCs. Skupiny po 15-ti, 25-ti nebo 50-ti COCs (15x, 25x, 50x) byly propláchnuty v PBS-PVA (0,01%) a následně podrobeny enzymatické digesci za účelem změření zadržené HA v COCs po 48 hod. *in vitro* kultivace. Mezi skupinami 15x a 25x COCs nebyly prokázány statisticky významné rozdíly. Signifikantní nárůst byl zaznamenán u vzorků s 50x COCs, kde HA vykazovala téměř 4-násobně vyšší koncentraci (213,58 ± 82,15 vs. 854,83 ± 113,47 μg/ml pro vzorky 25x a 50x COCs). Získaná data jsou shrnuta na Obr. 4.



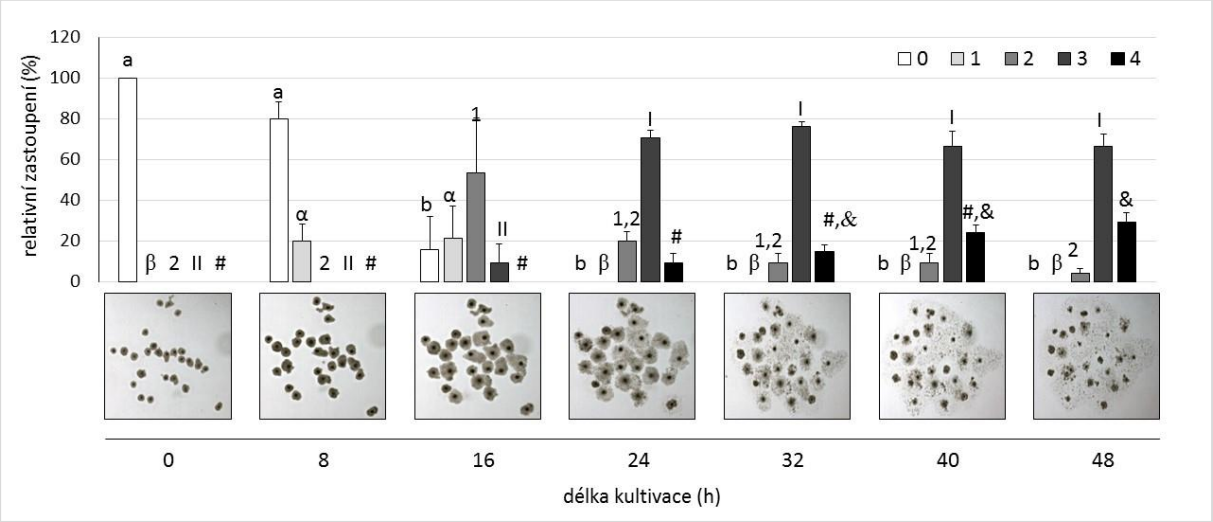
Obrázek 4) Závislost množství hyaluronové kyseliny na počtu kumulo-oocytárních komplexů.

Ověření závislosti obsahu HA na množství COCs po 48 hod. kultivace in vitro*. Vzorky byly připraveny za použití digesce SHH v PBS-PVA přes noc při teplotě 39°C v humidované atmosféře. Absorbance byla měřena při vlnové déllce 216 nm a koncentrace HA (μg/ml) ve vzorcích byla spočítána za využití polynomické křivky HA standardů, štěpených za stejných podmínek. Sloupce ukazují průměr 3 nezávislých experimentů ± SEM;* ^{a,b}*odlišné superskripty značí statisticky významné rozdíly (P < 0,05).*

SHH = hyaluronan lyáza ze Streptomyces hyalurolyticus*, PBS-PVA = 0,01% polyvinyl alkohol ve fosfátovém pufru*.

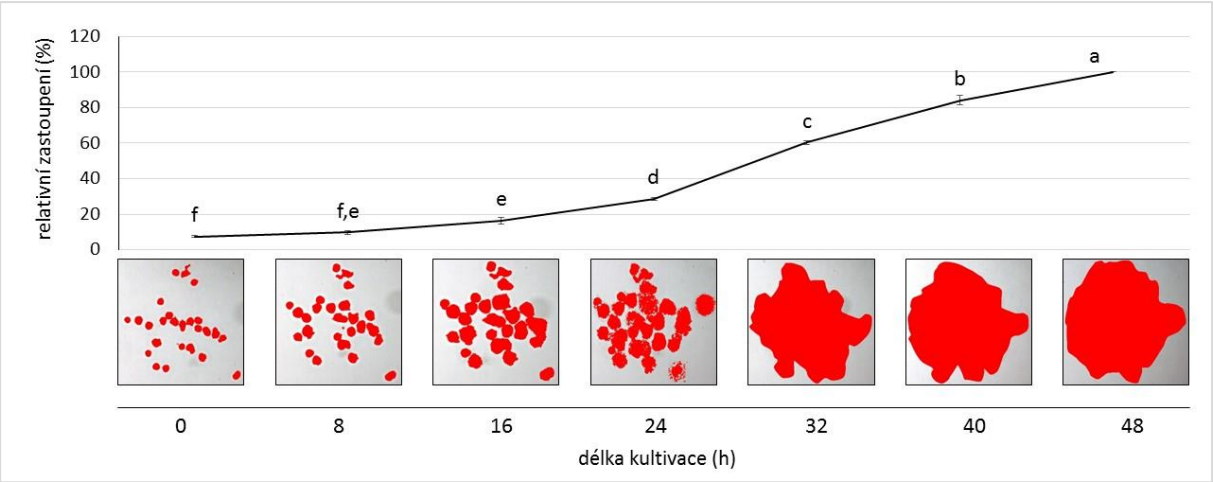
5.1.4 Porovnání jednotlivých metod hodnocení kumulární expanze

Testovány byly tři odlišné metody pro hodnocení kumulární expanze: (1) klasifikace expandovaného kumulu, (2) měření plochy expandovaného kumulu a (3) spektrofotometrická analýza HA. Všechny použité metody prokázaly narůstající trend v průběhu *in vitro* kultivace COCs (Obr 5 – 7). Klasifikace kumulu a měření plochy prokázaly první signifikantní nárůst po 16-ti hodinách kultivace (Obr. 5 a 6). Naproti tomu nebyl detekován rozdíl v obsahu HA a signifikantní nárůst nebyl naměřen ani mezi 16 a 24 hod. (Obr. 7). Signifikantní nárůst v produkci HA byl pozorován mezi 40 – 48 hod., kdy metoda klasifikace kumulu rozdíly nezaznamenala. Současně byla provedena kontrola meiotického zrání oocytů, kdy byl pozorován jeho standardní průběh (Tab. 1).



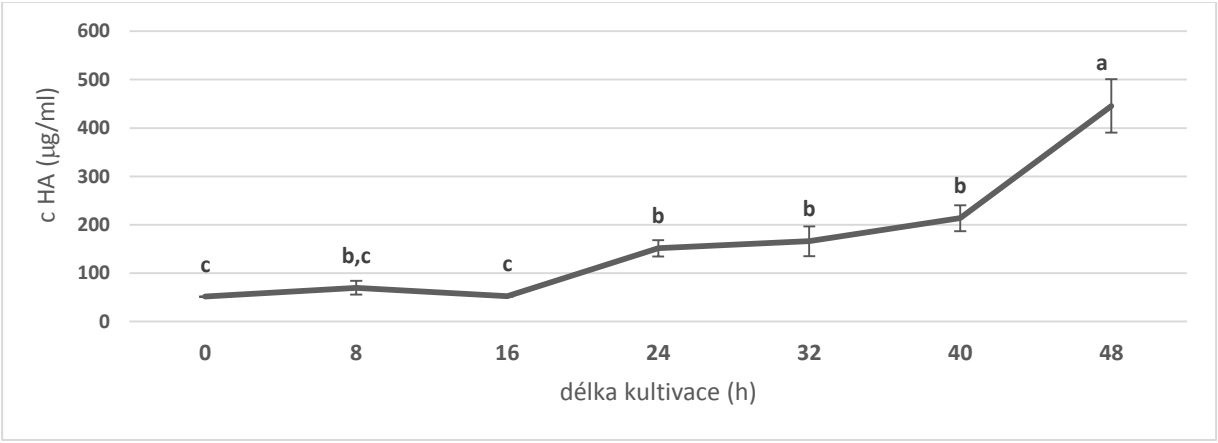
Obrázek 5) Vizuální klasifikace expandovaných kumulo-oocytárních komplexů.

Vizuální hodnocení expandovaných COCs bylo provedeno v 8-hodinových intervalech po dobu 48 hod (stupeň 0 = bez pozorovatelné expanze, +1 = minimální pozorovatelná expanze, +2 = expanze rozšířená v několika vrstvách, +3 = kompletní expanze mimo *vrstvy* corona radiata*, +4 = kompletní expanze včetně vrstvy* corona radiata*. Současně jsou uvedeny reprezentativní obrázky jednotlivých časových intervalů. Data jsou vyjádřena jako percentuální část populace COCs, sloupce ukazují průměr 3 nezávislých experimentů ± SEM;* $^{a,A;1,I;\alpha,\beta,\#,\&}$*odlišné superskripty značí statisticky významné rozdíly (P < 0,05).*



Obrázek 6) Měření plochy expandovaného kumulu.

Měření plochy expandovaného kumulu bylo provedeno v 8-hodinových intervalech po dobu 48 hod. Současně jsou uvedeny reprezentativní obrázky prahových hodnot COCs jednotlivých časových intervalů. Data jsou vztažena k COCs po 48-hodinové kultivaci (100 %), body ukazují průměry 3 nezávislých experimentů ± SEM; ^{a,b,c,d,e,f} *odlišné superskripty značí statisticky významné rozdíly (P < 0,05).*



Obrázek 7) Spektrofotometrická analýza HA.

Analýza HA zadržená v COCs byla provedena v 8-hodinových intervalech po dobu 48 hod. Vzorky COCs byly připraveny za použití digesce SHH v PBS-PVA, přes noc. Absorbance byla měřena při vlnové délce 216 nm a koncentrace HA (µg/ml) ve vzorcích byla spočítána za využití polynomické křivky HA standardů, štěpených za stejných podmínek. Body ukazují průměry 3 nezávislých experimentů ± SEM; ^{a,b,c} *odlišné superskripty značí statisticky významné rozdíly (P < 0,05).*

SHH = hyaluronan lyáza ze Streptomyces hyalurolyticus, *PBS-PVA = 0,01% polyvinyl alkohol ve fosfátovém pufru.*

Tabulka 1) Meiotické zrání oocytů v průběhu *in vitro* kultivace kumulo-oocytárních koplexů.

	0 h	8 h	16 h	24 h	32 h	40 h	48 h
GV	94.0 ± 8.5^{a}	96.8 ± 3.9^{a}	58.7 ± 11.6^{b}	3.0 ± 2.2^{c}	1.3 ± 1.3^{c}	-^{c}	-^{c}
LD	-^{b}	3.2 ± 0.9^{b}	22.0 ± 6.1^{a}	1.0 ± 0.5^{b}	-^{b}	-^{b}	-^{b}
MI	-^{b}	-^{b}	15.7 ± 5^{b}	91.0 ± 3.8^{a}	42.7 ± 20.5^{a,b}	29.5 ± 15.3^{b}	4.0 ± 1.4^{b}
AITI	-^{c}	-^{c}	-^{c}	5.0 ± 5.0^{b}	19.3 ± 5.0^{a}	14.9 ± 7.5^{a}	4.5 ± 2.3^{b}
MII	-^{c}	-^{c}	-^{c}	-^{c}	36.7 ± 20.8^{b}	49.2 ± 19.9^{b}	90.5 ± 4.2^{a}
Dg	6.0 ± 2.6^{a}	-^{a}	3.6 ± 3.3^{a}	-^{a}	-^{a}	6.4 ± 5.3^{a}	1.0 ± 0.4^{a}
počet	75	200	200	250	150	250	250

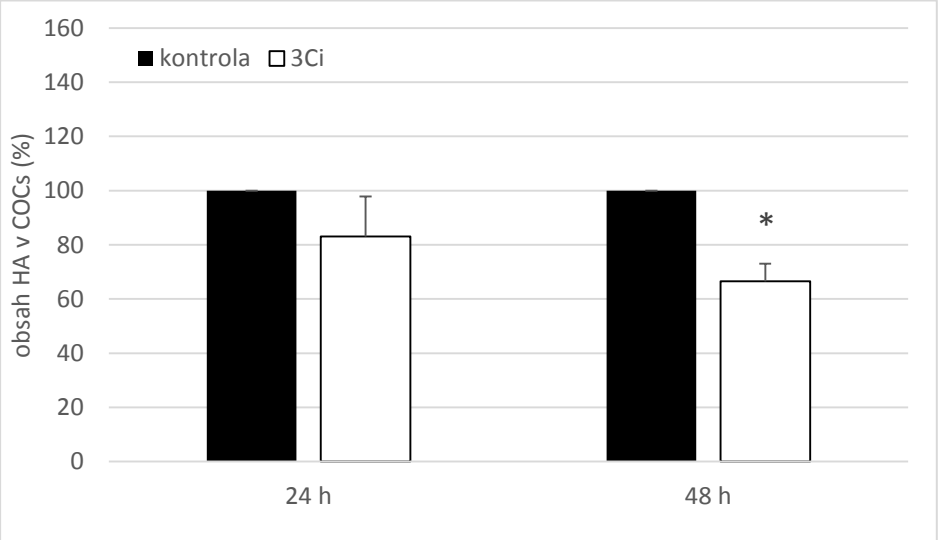
GV = zárodečný váček, LD = pozdní diakineze, MI = metafáze I, AI/TI = přechod anafáze I / telofáze I, MII = metafáze II. Data jsou vyjádřena jako percentuální zastoupení jednotlivých stádií meiózy a představují průměry minimálně tří nezávislých experimentů ± SEM, ^{a,b,c} odlišná písmena značí statisticky významné rozdíly ($P < 0,05$).

5.2 Testování vybraných faktorů na průběh kumulární expanze *in vitro*.

5.2.1 Vliv gasotransmiteru sulfanu na kumulární expanzi prasečích kumulo-oocytárních komplexů *in vitro*.

Cílem experimentu bylo otestovat vliv inhibice endogenní produkce sulfanu na kumulární expanzi a zrání prasečích oocytů *in vitro*. COCs byly ošetřeny trojkombinací inhibitorů (3Ci – kyselina oxamová, DL-propargylglycin a kyselina α-ketoglutarová sulfan) sulfan uvolňujících enzymů (CBS, CSE, resp. MPST) a kultivovány po dobu 24 a 48 hodin. Statisticky významné rozdíly v produkci HA byly prokázány po 48 hod. kultivace, kdy došlo u pokusné skupiny k poklesu produkce HA o 33,5 %. Rozdíly po 24 hod. kultivaci nebyly statisticky významné. Data jsou shrnuta na Obr. 8.

Současně byla provedena kontrola meiotického zrání oocytů po 48 hod. kultivace, při které byl pozorován statisticky významný pokles oocytů schopných dosáhnout stádia metafáze II, přechodu anafáze I / telofáze I i metafáze I. (viz Tabulka 2).



Obrázek 8) Efekt trojkombinace inhibitorů sulfan uvolňujících enzymů na produkci HA v průběhu kumulární expanze.

*Obsah HA byl měřen jako marker kumulární expanze po 24 a 48 hod. kultivace. COCs byly kultivovány v přítomnosti trojkombinace inhibitorů sulfan uvolňujících enzymů – 2,0 mM kyselina oxamová, 2,0 mM DL-propargylglycin a 5,0 mM kyselina α-ketoglutarová. Vzorky byly připraveny za použití digesce SHH v PBS-PVA, přes noc při teplotě 39°C v humidované atmosféře. Absorbance byla měřena při vlnové déllce 216 nm a koncentrace HA (μg/ml) ve vzorcích byla spočítána za využití polynomické křivky HA standardů, štěpených za stejných podmínek. Výsledek byl vyjádřen relativně ke kontrole neošetřených MI a MII oocytů. Sloupce ukazují průměr 3 nezávislých experimentů ± SEM; *superskripty značí statisticky významné rozdíly (P < 0,05).*

SHH = hyaluronan lyáza ze Streptomyces hyalurolyticus, PBS-PVA = 0,01% polyvinyl alkohol ve fosfátovém pufru

Tabulka 2) Efekt trojkombinace inhibitorů sulfan uvolňujících enzymů na meiotické zrání prasečích oocytů po 48 hod. kultivace *in vitro*.

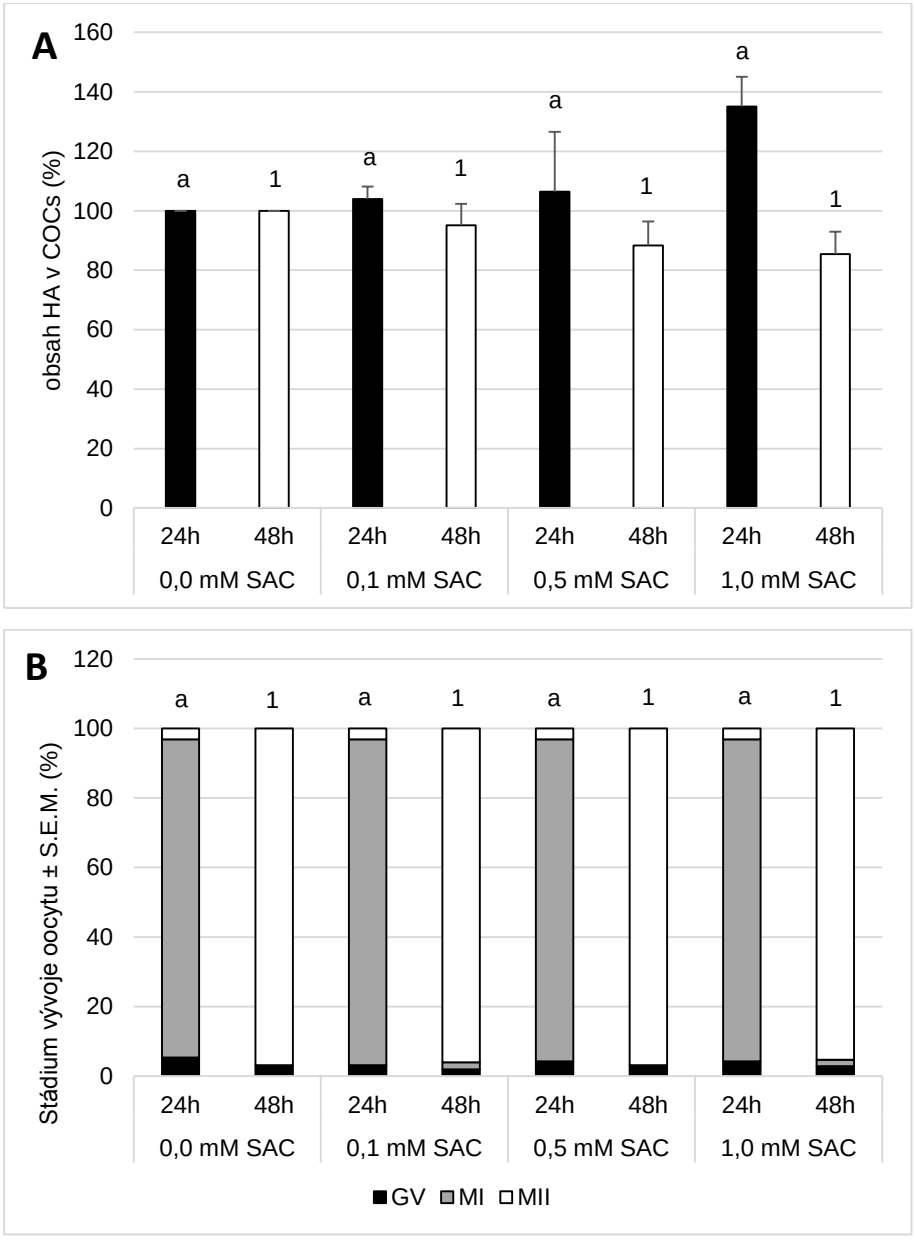
	kontrola	trojkombinace inhibitorů
GV	-	-
LD	-	-
MI	$0{,}5 \pm 1{,}1^{a}$	$20{,}8 \pm 5{,}2^{b,c}$
AITI	$4{,}8 \pm 1{,}9^{a}$	$14{,}2 \pm 1{,}4^{d}$
MII	$94{,}7 \pm 1{,}4^{a}$	$65{,}0 \pm 5{,}0^{d}$
počet	120	120

Výsledky meiotického zrání oocytů ošetřených trojkombinací inhibitorů sulfan uvolňujících enzymů (2 mM propargylglycin +2 mM oxamová kys.+ 5 mM ketoglutarová kys.) a kontroly po 48 hod. kultivace. GV = zárodečný váček, LD = pozdní diakineze, MI = metafáze I, AI/TI = přechod anafáze I / telofáze I, MII = metafáze II. Data jsou vyjádřena jako percentuální zastoupení jednotlivých stádií meiózy a představují průměry minimálně tří nezávislých experimentů ± SEM, ^{a,b,c,d} odlišná písmena značí statisticky významné rozdíly ($P < 0{,}05$).

5.2.2 Vliv česnekového derivátu S-allyl cysteinu na kumulární expanzi prasečích kumulo-oocytárních komplexů *in vitro*.

Cílem experimentu bylo otestovat vliv česnekového derivátu S-allyl cysteinu (SAC) na kumulární expanzi a zrání prasečích oocytů *in vitro*. Pokusné skupiny COCs byly ošetřeny za použití SAC o výsledných koncentracích 0,1 mM; 0,5 mM a 1,0 mM a následně *in vitro* kultivovány po dobu 24 a 48 hod. U žádné z použitých koncentrací nevykazovala produkce HA statisticky významné rozdíly. Výsledky jsou uvedeny na Obr. 9A.

Současně byla provedena kontrola meiotického zrání po 24 a 48 hod. kultivace, kdy rovněž nebyly prokázány statisticky významné rozdíly u žádné z testovaných koncentrací SAC (viz. Obr. 9B)



Obrázek 9) Efekt SAC na kumulární expanzi a meiotické zrání prasečích oocytů *in vitro*.

(A) Efekt SAC na produkci HA v COCs prasete po 24 a 48 hod. kultivace in vitro. *COCs byly kultivovány v přítomnosti SAC o koncentracích 0,1 mM; 0,5 mM a 1,0 mM. Vzorky byly připraveny za použití digesce SHH v PBS-PVA, přes noc při teplotě 39°C v humidované atmosféře. Absorbance byla měřena při vlnové déllce 216 nm a koncentrace HA (µg/ml) ve vzorcích byla spočítána za využití polynomické křivky HA standardů, štěpených za stejných podmínek. Výsledek byl vyjádřen relativně ke kontrole neošetřených MI a MII oocytů. Sloupce ukazují průměr 3 nezávislých experimentů ± SEM;* $^{a,1;b,2}$*odlišné superskripty značí statisticky významné rozdíly (P < 0,05).*

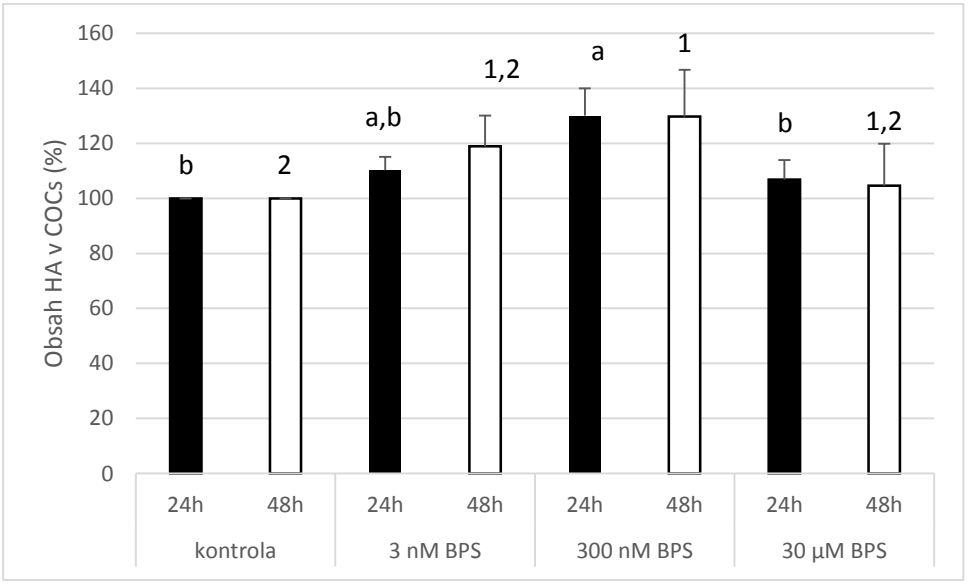
SHH = hyaluronan lyáza ze Streptomyces hyalurolyticus, *PBS-PVA = 0,01% polyvinyl alkohol ve fosfátovém pufru*.

(B) Efekt SAC na jaderné zrání prasečích oocytů po 24 a 48 hod. kultivaci in vitro. *GV = zárodečný váček, MI = metafáze I, MII = metafáze II. Sloupce ukazují průměr 3 nezávislých experimentů ± SEM;* $^{a,1;b,2}$*odlišné superskripty značí statisticky významné rozdíly (P < 0,05).*

5.2.3 Vliv endokrinního disruptoru Bisfenolu S na kumulární expanzi prasečích kumulo-oocytárních komplexů *in vitro*.

Cílem experimentu bylo otestovat vliv endokrinního disruptoru Bisfenolu S (BPS) na kumulární expanzi a zrání prasečích oocytů *in vitro*. Pokusné skupiny COCs byly ošetřeny za použití BPS o výsledných koncentracích 3,0 nM; 300 nM a 30 μM. COCs byly *in vitro* kultivovány po dobu 24 a 48 hod. Statisticky významný nárůst v produkci HA byl zaznamenán u koncentrace 300 nM BPS po 24 i 48 hod. kultivaci *in vitro*. U ostatních testovaných koncentrací nebyl rozdíl statisticky významný. Výsledky jsou uvedeny na Obr. 10.

Současně byla provedena kontrola meiotického zrání po 24 a 48 hod. kultivace, kdy došlo u všech testovaných koncentrací BPS ke statisticky významnému snížení počtu oocytů schopných dosáhnout MI fáze po 24 hod. kultivace a MII fáze po 48 hod. kultivace (viz. Tab 3 a 4).



Obrázek 10) Efekt BPS na kumulární expanzi prasečích COCs *in vitro*.

Efekt BPS na produkci HA v COCs prasete po 24 a 48 hod. kultivace in vitro*. COCs byly kultivovány v přítomnosti BPS o koncentracích 3,0 nM; 300 nM a 30 μM. Vzorky byly připraveny za použití digesce SHH v PBS-PVA, přes noc při teplotě 39°C v humidované atmosféře. Absorbance byla měřena při vlnové déllce 216 nm a koncentrace HA (μg/ml) ve vzorcích byla spočítána za využití polynomické křivky HA standardů, štěpených za stejných podmínek. Výsledek byl vyjádřen relativně ke kontrole neošetřených MI a MII oocytů. Sloupce ukazují průměr 3 nezávislých experimentů ± SEM;* $^{a,1;b,2}$*odlišné superskripty značí statisticky významné rozdíly (P < 0,05).*

SHH = hyaluronan lyáza ze Streptomyces hyalurolyticus*, PBS-PVA = 0,01% polyvinyl alkohol ve fosfátovém pufru*.

Tabulka 3) Efekt BPS na meiotické zrání prasečích oocytů po 24 hod. kultivace *in vitro.*

	kontrola	3 nM	300 nM	30 μM
GV	-[a]	-[a]	14,2 ± 0,5[b]	17,5 ± 0,5[c]
LD	-[a]	21,7 ± 0,6[b]	16,7 ± 0,6[b]	19,2 ± 0,8[b]
MI	100 ± 0,0[a]	78,3 ± 0,6[b]	69,2 ± 0,9[c]	63,3 ± 0,9[d]
AITI	-	-	-	-
MII	-	-	-	-
počet	120	120	120	120

Výsledky meiotického zrání oocytů ošetřených BPS (3,0 nM; 300 nM a 30 μM) a kontroly po 24 hod. kultivace. GV = zárodečný váček, LD = pozdní diakineze, MI = metafáze I, AI/TI = přechod anafáze I / telofáze I, MII = metafáze II. Data jsou vyjádřena jako percentuální zastoupení jednotlivých stádií meiózy a představují průměry minimálně tří nezávislých experimentů ± SEM, [a,b,c,d] odlišná písmena značí statisticky významné rozdíly (P < 0,05).

Tabulka 4) Efekt BPS na meiotické zrání prasečích oocytů po 48 hod. kultivace *in vitro.*

	kontrola	3 nM	300 nM	30 μM
GV	-	-	-	-
LD	-	-	-	-
MI	-[a]	5,8 ± 0,5[b]	13,3 ± 0,4[c]	36,7 ± 0,4[d]
AITI	-[a]	2,5 ± 0,5[a]	-[a]	-[a]
MII	100 ± 0,0[a]	91,7 ± 0,5[b]	86,7 ± 0,4[c]	63,3 ± 0,4[d]
počet	120	120	120	120

Výsledky meiotického zrání oocytů ošetřených BPS (3,0 nM; 300 nM a 30 μM) a kontroly po 48 hod. kultivace. GV = zárodečný váček, LD = pozdní diakineze, MI = metafáze I, AI/TI = přechod anafáze I / telofáze I, MII = metafáze II. Data jsou vyjádřena jako percentuální zastoupení jednotlivých stádií meiózy a představují průměry minimálně tří nezávislých experimentů ± SEM, [a,b,c,d] odlišná písmena značí statisticky významné rozdíly (P < 0,05).

6 Diskuze

Cílem disertační práce bylo ověřit hypotézu, že hyaluronová kyselina (HA) je markerem kumulární expanze odpovídající stádiu meiotického zrání a kvalitě oocytu. Pro tento účel byla vyvinuta metoda analytického stanovení HA v kumulo-oocytárních komplexech savců. Metoda je založena na spektrofotometrickém stanovení koncentrace štěpných produktů β-eliminace HA, přičemž byla následně ověřena a zavedena do experimentů.

V pokusech zaměřujících se na zavedení a optimalizaci metody spektrofotometrie byla jako optimální média určena fosfátový pufr PBS, popř. PBS obohacené o 0,01% PVA, které je z hlediska manipulace s COCs výhodnější. Naopak za nevhodná média lze označit původní kultivační média. Jejich nevhodnost se odvíjí od širokého spektra nízko- i vysokomolekulárních látek, které absorbují světlo v obdobných vlnových délkách jako štěpné produkty HA. Toto sledování je v souladu s naměřenými absorbcemi pro glukózu a albumin (Glazer *et al*., 1963; Albalasmeh *et al*., 2013), které v kultivačním médiu plní úlohu zdroje energie, resp. přenosu těchto látek. Ke štěpení polymeru HA byla použita vysoce specifická rekombinantní lyáza produkována *Streptomyces hyalurolyticus* (SHH; Eppig, 1980; Vanderhyden, 1993), která k enzymatické digesci využívá chemické reakce β-eliminace za vzniku dvojných vazeb. Pouze dvojné vazby v dimerech a oligomerech HA vzniklé štěpením SHH jsou schopné absorpce v UV oblasti (Alkrad *et al*., 2003). Naopak, za zcela nevhodnou byla označena hyaluronidáza izolovaná z bovinních testis, která štěpí polymer HA hydroliticky, kdy nedochází k vzniku dvojné vazby (Saitoh *et al*., 1995). Použitím této hyaluronidázy jako negativní kontroly bylo ověřeno, že naměřená absorbce po použití lyázy SHH odpovídala vzniku dvojných vazeb.

Při spektrofotometrické analýze štěpných produktů HA po digesci SHH byly zjištěny nižší hodnoty absorbance ve srovnání se standardy neštěpených oligomerů 4 – 6 HA (jednotek heterodimerů). Neštěpené oligomery dlouhé 8 – 10 HA opakování vykazovaly srovnatelné hodnoty absorbance. Toto pozorování, podpořené HPLC analýzou (nepublikovaná data), naznačuje, že při digesci HA polymeru prostřednictvím SHH dochází k mírným ztrátám oproti neštěpeným polymerům o délce 4 – 6 HA opakování. Dalším vysvětlením je nižší efektivita lyázy SHH ve štěpení oligomerů, kdy většina štěpných produktů zůstává v délce 8 – 10 HA. Tyto skutečnosti mají za následek mírné snížení detekčního limitu štěpeného HA polymeru v porovnání se standardy krátkých oligomerů (tj. 4 – 6 HA). Minimální detekovatelná koncentrace produktů β−eliminace pro

spektrofotometrii tak byla určena jako 60 μg/ml HA polymeru, která odpovídá hodnotě absorbance ~0.13.

Součástí optimalizace metody byla rovněž optimalizace vlnové délky použité pro analýzu produktů HA. Optimální vlnová délka v našich laboratorních podmínkách byla stanovena na 216 nm a následně použita v dalších experimentech. Stanovená vlnová délka je srovnatelná s vlnovou délkou používanou v laboratoři partnerského pracoviště (Dr. Pavel Klein, pers. comm.). V publikovaných studiích jsou pro spektrofotometrické měření produktů HA digesce popisovány nepatrně odlišné vlnové délky (Takagaki *et al*., 1994; Alkrad *et al*., 2003), což poukazuje na rozdíly v laboratorních podmínkách. Z výše uvedených informací plyne nutnost vždy vlnovou délku optimalizovat pro příslušné laboratorní podmínky a použitý přístroj.

Cílem dalšího experimentu bylo ověřit, zda je HA v kumulo-oocytárním komplexu (COCs) prasete výše popsanou metodou měřitelná a použitelná jako spolehlivý ukazatel kumulární expanze. Pro tento účel byly použity vzorky s různým počtem COCs, které byly 3x opláchnuty v PBS-PVA. Tímto experimentem byla prokázána závislost koncentrace HA na množství COCs, kdy HA zadržená v COCs byla signifikantně zvýšena ve vzrorcích obsahujících 50 COCs. Ačkoliv mezi vzorky obsahujícími 15 a 25 COCs nebyly v obsahu HA pozorovány statisticky významné rozdíly, vzorky s 25 COCs poskytují přesnější analýzu s ohledem na variabilitu COCs. Uvedené výsledky dokazují, že použitá metoda je schopná analyzovat nejen chemicky čistý produkt HA, ale rovněž HA izolovanou z biologické matrice, kterou je v tomto experimentu prasečí COCs, připravené v již dříve ověřeném pufru PBS-PVA. Výsledek experimentu dokázal, že produkty spektrofotometrického měření po digesci HA odpovídají produktům β-eliminace, které v reálných vzorcích COCs odrážejí obsah HA.

V našich experimentech byla porovnávána HA analýza s běžně používanými metodami hodnocení kumulární expanze – subjektivní klasifikací COCs (podle Vanderhyden *et al*., 1990) a měřením plochy expandovaného kumulu (podle Daen *et al*., 1990). Jako nejvíce konzistentní se jeví metoda měření plochy expandovaného kumulu. Na druhou stranu tato neinvazivní jednoduchá metoda není schopna postihnout trojrozměrnou strukturu expandovaného kumulu, která může být v průběhu *in vitro* kultivace ovlivněna řadou sloučenin a celkovými kultivačními podmínkami.

Trojrozměrnou strukturu COCs respektují metody založené na měření komponent expandovaného kumulu, často nejhojněji zastoupenou HA. Pro tento účel byla navržena metoda radioaktivního značení prekurzorů HA syntézy (Eppig, 1979), v průběhu které jsou do kultivačního média aplikovány prekurzory HA označené radioizotopem, nejčastěji [^{3}H]glukosaminem. Po proběhlé kumulární expanzi je obsah HA kvantifikován pomocí radioaktivního signálu emitovaného prekurzory HA. Metoda analytického stanovení HA prostřednictvím spektrofotometrie spojuje

výhody metod založených na měření obsahu GAGs, čili objektivní hodnocení kumulární expanze, které je schopno postihnout 3D strukturu expandovaného kumulu. Současně odstraňuje jejich hlavní nevýhody, které spočívají zejména v práci s radioaktivním materiálem. Oproti radioizotopovým metodám je analytické měření HA založeno na reálné produkci HA, bez přídavku radioizotopově označených prekurzorů HA syntézy na začátku *in vitro* kultivace. Díky absenci radioizotopů je spektrofotometrická analýza rovněž dostupnější.

Kvalita kumulární expanze ovlivňuje meiotické zrání oocytu a jeho vývojovou kompetenci (Qian *et al*., 2003; Feuerstein *et al*., 2012; Bergandi *et al*., 2014). Současně kumulární expanze fyziologicky odráží stádium meiotického zrání oocytu (Procházka *et al*., 1998; Dragovic *et al*., 2005). V souladu s těmito doposud publikovanými poznatky jsou i naše výsledky, při kterých bylo znovuzahájení meiózy detekováno přibližně po 16 hod. *in vitro* kultivace, kdy je rovněž započata kumulární expanze. Toto pozorování je v souladu s pracemi, které popisují význam kumulární expanze pro zahájení meiózy oocytu (Dekel *et al*., 1981; Chen *et al*., 1990), stejně jako efekt CEEFs na intenzitu kumulární expanze (Nakayama *et al*., 1996).

HA samotná má funkci signální molekuly a ligandu, která se váže na receptory ze skupiny HABPs. Do HABPs se řadí např. receptor CD44, který se po vazbě HA účastní regulace průběhu meiotického zrání (Kimura *et al*., 2002; Yokoo *et al*., 2007). Po iniciaci kumulární expanze gonadotropinem nebo růstovým faktorem může být HA produkce zapojena v pozdějším meiotickém zrání (Abeydeera *et al*., 1998). V případě nedostatečné interakce HA a CD44 v průběhu *in vitro* kultivace dochází ke snížení úspěšnosti *in vitro* zrání, oplození i časného embryonálního vývoje (Yokoo *et al*., 2007). U žab rodu *Xenopus* se HA váže na iontové kanály v oocytech a podílí se na regulaci membránového potenciálu prostřednictvím toku iontů (Fraser, 1997). Na základě výčtu funkcí HA je zřejmé, že molekula HA představuje vhodný marker kvality kumulární expanze i meiotického zrání oocytu *in vitro*.

Po vývoji a ověření spektrofotometrické metody analýzy štěpných produktů HA byla metoda zavedena do experimentů, studujících signální molekuly gasotrasmiterů, látky s potenciálně pozitivním účinkem stejně jako polutanty ohrožující kvalitu COCs a oocytů.

Jakožto látka s potenciálně pozitivním účinkem byl testován gasotransmiter sulfan, u kterého lze předpokládat zapojení do regulace buněčného cyklu v průběhu meiotického zrání oocytu a růstu kumulu, které bylo popsáno u somatických buněk (Njie-Mbye *et al*., 2012), s přihlédnutím k poznatku, že v granulózních a kumulárních buňkách byla detekována exprese sulfan produkujícího enzymu CBS (Liang *et al*., 2006; Liang *et al*., 2007).

V našich experimentech byl v případě inhibice endogenní produkce sulfanu pozorován statisticky významný pokles v produkci HA o 33,5 %. Současně byl pozorován statisticky

významný pokles počtu oocytů schopných dosáhnout stádia metafáze II, přechodu anafáze I / telofáze I i metafáze I po 48 hod. kultivace. Je známo, že meiotické zrání může být urychleno dřívějším vzestupem hladin MPF a MAKP (Kubelka *et al.*, 2000). Akcelerace aktivity MPF a MAPK společně se zrychleným *in vitro* zráním oocytů po aplikaci donoru H_2S byla popsána na prasečích oocytech (Nevoral *et al.*, 2014). Doposud nebyl prokázán přímý efekt H_2S na MPF a MAPK a přesný mechanizmus účinku H_2S na MPF a MAPK není znám. Bylo popsáno, že H_2S ovlivňuje řadu faktorů prostřednictvím jejich přímé sulfhydratace (Mustafa *et al.*, 2009). Lze předpokládat, že H_2S působí na MPF a MAPK nepřímo prostřednictvím jiných molekul, např. iontových kanálů (Tang *et al.*, 2010) nebo nadřazených kináz (Hu *et al.*, 2008; Huang *et al.*, 2010), a že sulfhydratace těchto proteinů může následně ovlivňovat meiotické zrání oocytu. U somatických buněk byl popsán stimulační efekt H_2S na signální dráhy cAMP/PKA (Njie-Mbye *et al.*, 2012) a PI3K/Akt (Huang *et al.*, 2010), o kterých je známo, že se rovněž účastní regulace zrání oocytu (Wassarman, 1988; Kalous *et al.*, 2009).

Dále byl testován česnekový derivát S-allyl cystein (SAC). Česnekové deriváty jsou známy svými antioxidačními účinky na živočišné buňky (Banerjee *et al.*, 2001), ve kterých vychytávají volné radikály (Prasad *et al.*, 1996). Mechanizmus působení česnekových derivátů spočívá pravděpodobně v jejich zapojení do signálních kaskád produkce sulfanu. V experimentech provedených na somatických buňkách bylo prokázáno, že česnekové deriváty stimulují aktivitu sulfan produkujících enzymů a tím produkci H_2S, který následně působí antioxidačně a antiapoptoticky (Louis *et al.*, 2012; Tsai *et al.*, 2015; Chen *et al.*, 2016). V našich experimentech nebyl prokázán statisticky významný rozdíl v obsahu HA v COCs ani v meiotickém zrání oocytů po ošetření SAC. Dvořáková *et al.* (2016) rovněž popisuje, že SAC neovlivňuje MFP ani MAPK.

Výše uvedené výsledky naznačují, že stanovení HA produkce může být úspěšně použito jako marker *in vitro* zrání oocytů. Odlišná situace byla zjištěna u testování účinku BPS na kumulární expanzi a *in vitro* zrání prasečích oocytů. V případě *in vitro* zrání došlo u všech testovaných koncentrací BPS ke statisticky významnému snížení počtu oocytů schopných dosáhnout MI fáze po 24 hod. kultivace a MII fáze po 48 hod. kultivace. Naopak v případě hodnocení kumulární expanze výsledky prokázaly, že BPS je schopen zvýšit množství zadržené HA v COCs. Tento efekt byl pozorován po 24 i 48 hodinové kultivaci *in vitro* v koncentraci 300 nM BPS. Je známo, že BPS je schopen v organizmu napodobovat účinky estrogenů (Michalowicz *et al.*, 2015). V případě myších oocytů bylo popsáno, že 17β–estradiol je zodpovědný za adekvátní expanzi kumulu a rovněž expresi normální hladiny mRNA pro hyaluronan-syntetizující enzym HAS2 *in vitro* (Sugiura *et al.*, 2010). Lze tedy předpokládat, že prostřednictvím estrogenního efektu BPS dochází ke stimulaci hyaluronan-syntetizujících enzymů a tím ke stimulaci produkce HA.

Z dostupných informací odborné literatury můžeme naše výsledky porovnat s BPA, který má rovněž negativní dopad na *in vitro* zrání oocytů (Can *et al*., 2005, Wang *et al*., 2016). BPA je také schopný měnit množství HA a vyvolávat naopak pokles zadržené HA v prasečích COCs (Mlynarčíková *et al*., 2009) a potlačit kumulární expanzi v prasečích COCs (Wang *et al*., 2016). Odlišný efekt BPS lze vysvětlit rozdílným mechanismem působení BPS v kumulárních buňkách.

Naše experimenty prokázaly, že spektrofotometrická metoda stanovení štěpných produktů HA prostřednictvím β−eliminace je vhodná pro hodnocení expanze prasečích COCs, která v reálných vzorcích odráží skutečný obsah HA. Detekční limit metody je stanoven na koncentraci 60 μg/ml HA polymeru, odpovídající hodnotě absorbance ~0.13. Na základě našich zkušeností doporučujeme před měřením reálných vzorků metodu optimalizovat proměřením koncentrační řady HA standardu v UV spektru pro zjištění optimální vlnové délky, která se v závislosti na příslušných laboratorních podmínkách může lišit.

Alternativní metodou spektrofotometrického stanovení zadržené HA v COCs by se mohla stát HPLC analýza, díky které bychom mohli dosáhnout vyšší citlivosti detekce HA oligomerů. Současně by tato metoda mohla využít širšího spektra enzymů štěpících HA polymer, jelikož není vázaná na detekci dvojných vazeb. Do budoucna by bylo rovněž vhodné zaměřit experimenty na vývoj metody, která by zajistila analýzu HA uvolněné do kultivačního média.

7 Závěr

Kumulární expanze kumulo-oocytárního komplexu a meiotické zrání oocytu představují klíčové procesy pro oblast reprodukčních biotechnologií. V průběhu meiotického zrání oocytu dochází ke kumulární expanzi, kdy je prostřednictvím kumulárních buněk produkována extracelulární matrix bohatá na hyaluronovu kyselinu (HA).

Doposud používané metody hodnocení kumulární expanze skýtají řadu úskalí, která plynou z jejich nepřesnosti (v případě vizuálních metod) či v náročnosti na jejich dostupnost (v případě metod radioizotopových a imunologických). Nové metody, které by umožnily detailnější a dostupnější studium těchto procesů, najdou uplatnění jak v základním vědeckém výzkumu, tak v odborné praxi. Byla stanovena hypotéza, že analytické stanovení hyaluronové kyseliny pomocí měření produktů β-eliminace v *in vitro* kultivovaných COCs může sloužit jako marker kumulární expanze, odpovídající stádiu meiotického zrání oocytu.

Na základě optimalizačních experimentů a ověření detekce produktů β-eliminace byla zavedena spektrofotometrická metoda stanovení HA, která byla následně porovnána s vizuálními metodami hodnocení kumulární expanze. Bylo zjištěno, že metoda spektorofotometrického stanovení HA v COCs odráží skutečný obsah HA v reálných vzorcích COCs, může sloužit jako marker meiotického zrání oocytů a představuje vhodnou metodu pro hodnocení kumulární expanze prasečích COCs *in vitro*.

V průběhu testování vlivu vybraných faktorů na kumulární expanzi prasečích COCs spektrofotometrickou metodou bylo zjištěno, že gasotransmiter sulfan je zapojen jak v procesu meiotického zrání, tak kumulární expanze *in vitro*. Inhibice endogenní produkce sulfanu snižuje kumulární expanzi a rovněž zpomaluje *in vitro* zrání oocytů. Naopak česnekový derivát S-allyl cystein nemá na kumulární expanzi ani na *in vitro* zrání oocytů vliv. V případě endokrinního disruptoru BPS došlo k inhibici meiotického zrání oocytu a zároveň ke zvýšení množství HA v průběhu kumulární expanze. Lze předpokládat, že v tomto případě se uplatnila estrogenní aktivita BPS, která vedla ke stimulaci hyaluronan-syntetizujích enzymů a následně k navýšení množství HA v COCs.

Výsledky práce ukazují, že spektrofotometrická metoda stanovení produktů β-eliminace představuje přesnou a vhodnou metodu pro hodnocení kumulární expanze, která nabízí využití

nejen pro základní vědecký výzkum, ale také praktické využití pro šlechtění a asistovanou reprodukci, kde zajistí kvalitativní selekci oocytů pro biotechnologické postupy.

8 Seznam použité literatury

Abeydeera, L. R., Wang, W. H., Cantley, T. C., Prather, R. S., Day, B. N. 1998: Presence of beta-mercaptoethanol can increase the glutathione content of pig oocytes matured in vitro and the rate of blastocyst development after in vitro fertilization. Theriogenology. 50(5). 747–56.

Albalasmeh, A. A., Berhe, A. A., Ghezzehei, T. A. 2013. A new method for rapid determination of carbohydrate and total carbon concentrations using UV spectrophotometry. Carbohydrate Polymers. 97(2). 253-61.

Alberts, B., Bray, D., Johnson, A., Lewis, J., Raff, M., Roberts, K., Walter, P. 1998. Základy buněčné biologie. Espero Publishing s.r.o. Ústí nad Labem. p. 630. ISBN: 8090290620.

Alexandreanu, I. C., Lawson, D. M. 2003. Heme oxygenase in the rat ovary: immunohistochemical localization and possible role in steroidogenesis. Experimental Biology and Medicine. 228(1). 59-63.

Alkrad, J. A., Mrestani, Y., Stroehl, D., Wartewig, S., Neubert, R. 2003. Characterization of enzymatically digested hyaluronic acid using NMR, Raman, IR, and UV-Vis spectroscopies. Journal of Pharmaceutical and Biomedical Analysis. 31(3). 545–50.

Amale, M. H., Shahne, Z., Abavisani, A., Nasrollahi, S. 2011. Effects of inhibiting nitric oxide synthase on cumulus expansion and nuclear maturation of sheep oocytes. Czech Journal of Animal Science. 56(6). 284–29.

Auclair, S., Uzbekov, R., Elis, S., Sanchez, L., Kireev, I., Lardic, L., Dalbies-Tran, R., Uzbekova, S. 2013. Absence of cumulus cells during in vitro maturation affects lipid metabolism in bovine oocytes. American Journal of Physiology-Endocrinology and Metabolism. 304(6). E599-E613.

Bainbridge, S. A., Smith, G. N. 2005. HO in pregnancy. Free Radical Biology and Medicine. 38(8). 979-88.

Banerjee, S. D., Toole, B. P. 1992. Hyaluronan-binding protein in endothelial cell morphogenesis. The Journal of Cell Biology. 119(3). 643-52.

Banerjee, S. K., Maulik, M., Manchanda, S. C., Dinda, A. K., Das, T. K., Maulik, S. K. 2001. Garlic-induced alteration in rat liver and kidney morphology and associated changes in endogenous antioxidant status. Food and Chemical Toxikology. 39(8). 793-97.

Banerjee, S. K., Mukherjee, P. K., Maulik, S. K. 2003. Garlic as an antioxidant: the good, the bad and the ugly. Phytotherapy Research. 17(2). 97-106.

Bartolazzi, A., Nocks, A., Aruffo, A., Spring, F., Stamenkovic, I. 1996. Glycosylation of CD44 is implicated in CD44-mediated cell adhesion to hyaluronan. The Journal of Cell Biology. 132(6). 1199-1208.

Ben-Jonathan, N., Steinmetz, R. 1998. Xenoestrogens: the emerging story of bisphenol A. Trends in Endocrinology & Metabolism. 9(3). 124-28.

Bergandi, L., Basso, G., Evangelista, F., Canosa, S., Dalmasso ,P., Aldieri, E., Revelli, A., Benedetto, C., Ghigo D. 2014. Inducible nitric oxide synthase and heme oxygenase 1 are expressed in human cumulus cells and may be used as biomarkers of oocyte competence. Reproductive Sciences. 21(11). 1370–77.

Bernhardt, M. L., Kim, A. L., O´Halloran, T. V., Woodruff, T. K. 2011. Zinc requirement during meiosis I – meiosis II transitiv in mouse oocytes is dependent of the MOS-MAPK pathway. Biology of Reproduction. 84(3). 526-36.

Bernhardt, ML., Kong, BY., Kim, AM., O´Halloran, TV., Woodruff, TK. 2012. A zinc-dependent mechanism regulates mestic progression in mammalian oocytes. Biology of Reproduction. 86(4). 114-18.

Bielanska-Osuchowska, Z. 2006. Oogenesis in pig ovaries during the prenatal period: ultrastructure and morphometry. Reprod Biol. 6(2). 161-193.

Bonnet, A., Le Cao, K. A., SanCristobal, M., Benne, F., Robert-Granie, C., Law-So, G., Fabre, S., Besse, P., De Billy, E., Quesnel, H., Hatey, F., Tosser-Klopp, G. 2008. *In vivo* gene expression in granulosa cells during pig terminal follicular development. Reproduction. 136(2). 211-24.

Borrelli, F., Capasso, R., Izzo, A. A. 2007. Garlic (Allium sativum L.): adverse effects and drug interactions in humans. Molecular Nutrition & Food Research, 51(11). 1386-97.

Bouton, M. C., Boulaftali, Y., Richard, B., Arocas, V., Michel, J. B., Jandrot-Perrus, M. 2012. Emerging role of serpinE2/protease nexin-1 in hemostasis and vascular biology. Blood. 119(11). 2452-57.

Buccione, R., Schroeder, A. C., Eppig, J. J. 1990. Interactions between somatic cells and germ cells throughout mammalian oogenesis. Biology of Reproduction. 43(4). 543-47.

Burnett, A. L. 2002. Nitric oxide regulation of penile erection: biology and therapeutic implications. Journal of Andrology. 23(5).

Can, A., Semiz, O., Cinar, O. 2005. Bisphenol-A induces cell cycle delay and alters centrosome and spindle microtubular organization in oocytes during meiosis. Molecular Human Reproduction. 11(6). 389-96.

Canipari, R., Epifano, O., Stracusa, G., Salustri, A. 1995. Mouse oocytes inhibic plasminogen activator production by ovarian cumulus and granulosa cells. Developmental Biology. 167(1). 371-78.

Cella, M., Farina, M. G., Sarmiento, M. I. K., Chianelli, M., Rosenstein, R. E., Franchi, A. M. 2006. Heme oxygenase–carbon monoxide (HO–CO) system in rat uterus: Effect of sexual steroids and prostaglandins. The Journal of Steroid Biochemistry and Molecular Biology. 99(1). 59-66.

Colborn, T., Vom Saal, F. S., Soto, A. M. 1993. Developmental effects of endocrine-disrupting chemicals in wildlife and humans. Environmental Health Perspectives. 101(5). 378.

Corzo-Martínez, M., Corzo, N., Villamiel, M. 2007. Biological properties of onions and garlic. Trends in Food Science & Technology. 18(12). 609-25.

Daen, F. P., Sato, E., Naito, K., Toyoda, Y. 1994. The effect of pig follicular fluid fractions on cumulus expansion and male pronucleus formation in porcine oocytes matured and fertilized in vitro. Journal of Reproduction and Fertility. 101(3). 667-73.

Davidoff, M. S., Middendorff, R., Mayer, B., DeVente, J., Koesling, D., Holstein, A. F. 1996. Nitric oxide/cGMP pathway components in the Leydig cells of the human testis. Cell and Tissue Research. 287(1). 161-70.

Dekel, N., Beers, W. H. 1980. Development of the rat oocyte in vitro: inhibition and induction of maturation in the presence or absence of the cumulus oophorus. Developmental Biology. 75(2). 247-54.

Dekel, N., Hillensjo, Y., Kraicer PF. 1979. Maturation effects of gonadotropins on the cumulus-oocyte complex of the rat. Biology of Reproduction. 20(2). 191-97.

Dekel, N., Lawrence, T. S., Gilula, N. B., Beers, W. H. 1981. Modulation of cell-to-cell communication in the cumulus-oocyte complex and the regulation of oocyte maturation by LH. Developmental Biology. 86(2). 356-62.

di Villa Bianca, R. D. E., Sorrentino, R., Maffia, P., Mirone, V., Imbimbo, C., Fusco, F., De Palma, R., Ignarro, L. J., Cirino, G. 2009. Hydrogen sulfide as a mediator of human corpus cavernosum smooth-muscle relaxation. Proceedings of the National Academy of Sciences. 106(11). 4513-18.

Dierich, A., Sairam, M. R., Monaco, L., Fimia, G. M., Gansmuller, A., LeMeur, M., Sassone-Corsi, P. 1998. Impairing follicle-stimulating hormone (FSH) signaling in vivo: targeted disruption of the FSH receptor leads to aberrant gametogenesis and hormonal imbalance. Proceedings of the National Academy of Sciences. 95(23). 13612-17.

Ding, W., Zhang, W., Hui, F. M., Zhang, Y. H., Zhang, F. F., Li, X. M., Shi, F. X. 2012. Cell-specific expression and immunolocalization of nitric oxide synthase isoforms and soluble guanylyl cyclase α 1 and β 1 subunits in the ovary of fetal, neonatal and immature pigs. Animal Reproduction Science. 131(3). 172-80.

Dragovic, R. A., Ritter, L. J., Schulz, S. J., Amato, F., Armstrong, D. T., Gilchrist, R. B. 2005. Role of oocyte-secreted growth differentiation factor 9 in the regulation of mouse cumulus expansion. Endocrinology. 146(6). 2798-806.

Dvořáková, M., Heroutová, I., Němeček, D., Adámková, K., Krejčová, T., Nevoral, J., Kučerová-Chrpová, V., Petr, J., Sedmíková, M. 2016. The antioxidative properties of S-allyl cysteine not only influence somatic cells but also improve early embryo cleavage in pigs. PeerJ, 4, e2280.

Ebeling, S., Topfer, D., Meinecke, B. 2011. Steroidogenesis and the influence of MAPK activity during in vitro maturation of porcine cumulus oocyte complexes. Reproduction in Domestic Animals. 46(3). 513-19.

Eicher, E. M., Washburn, L. L. 1986. Genetic kontrol of primary sex determination in mice. Annual Review of Genetics. 20(1). 327-60.

Eladak, S., Grisin, T., Moison, D., Guerquin, M. J., N'Tumba-Byn, T., Pozzi-Gaudin, S.,Benachi, A., Livera, G., Rouiller-Fabre, V., Habert, R. 2015. A new chapter in the bisphenol A story: bisphenol S and bisphenol F are not safe alternatives to this compound. Fertility and Sterility. 103(1). 11-21.

Ellis, I., Banyard, J., Schor, SL. 1997. Differential response of fetal and adult fibroblast to cytokines: cell migration and hyaluronan synthesis. Development. 124(8). 1593-600.

Elvin, J. A., Yan, C., Wang, P., Nishimori, K., Matzuk, M. M. 1999. Molecular characterization of the follicle defects in the growth differentiation factor 9 – deficient ovary. Molecular Endocrinology. 13(6). 1018-34.

Eppig, J. J. 1979. FSH stimulates hyaluronic acid synthesis by oocyte–cumulus cell complexes from mouse preovulatory follicles. Nature. 281(5731) 483-84.

Eppig, J. J. 1980. Role of FSH stimulated cumulus expansion by mouse oocytecumulus cell complexes in vitro. Biology of Reproduction. 22(3). 629-33.

Eppig, J. J. 2001. Oocyte control of ovarian follicular development and function in mammals. Reproductions.122(6). 829-38.

Eppig, J. J., Peters, A. H., Telfer, E. E., Wigglesworth, K. 1993. Production of cumulus expansion enabling factor by mouse oocytes grown in vitro: preliminary characterization of the factor. Molecular Reproduction and Development. 34(4). 450-56.

Eppig, J. J., Wigglesworth, K., Pendola, F., Hirao, Y. 1997. Murine oocytes suppress expression of luteinizing hormone receptor messenger ribonucleic acid by granulosa cells. Biology of Reproduction. 56(4). 976-84.

Fagbohun, C. F., Downs, S. M. 1990. Maturation of the mouse oocyte-cumulus cell complex: stimulation by lectins. Biology of Reproduction. 42(3). 413-23.

Fan H. Y., Sun Q. Y. 2004. Involvement of mitogen-activated protein kinase cascade during oocyte maturation and fertilization in mammals. Biology of Reproduction. 70(3). 535-47.

Fan, H. Y., Huo, L. J., Meng, X. Q., Zhong, Z. S., Hou, Y., Chen, D. Y., Sun, Q. Y. 2003. Involvement of calcium/calmodulin-dependent protein kinase II (CaMKII) in mestic maturation and activation of pig oocytes. Biology of Reproduction. 69(5). 1552-64.

Fan, H., Tong, CH., Chen, D., Sun, Q. 2002. Roles of MAP kinase signaling pathway in oocyte meiosis. Chinese Science Bulletin. 47(14). 1157-62.

Ferreira, E. M., Vireque, A. A., Adona, P. R., Meirelles, F. V., Ferriani, R. A., Navarro, P. A. 2009. Cytoplasmic maturation of bovine oocytes: structural and biochemical modifications and acquisition of developmental competence. Theriogenology. 71 (5). 836-48.

Feuerstein, P., Puard, V., Chevalier, C., Teusan, R., Cadoret, V., Guerif, F., Houlgatte, R., Royere, D. 2012. Genomic assessment of human cumulus cell marker genes as predictors of oocyte developmental competence: impact of various experimental factors. PLoS One. 7(7). e40449.

Fraser, S. P. 1997. Hyaluronan activates calcium-dependent chloride currents in Xenopus oocytes. FEBS Letters. 404(1). 56-60.

Fülöp, C., Kamath, R. V., Li, Y., Otto, J. M., Salustri, A., Olsen, B. R.,Glant, T.T., Hascall, V. C. 1997. Coding sequence, exon–intron structure and chromosomal localization of murine TNF-stimulated gene 6 that is specifically expressed by expanding cumulus cell–oocyte complexes. Gene. 202(1). 95-102.

Ganong, W. F. 2005. Přehled lékařské fyziologie, dvacáté vydání. Galén. p. 890. ISBN: 8072623117.

Ghasemi, M., Dehpour, A. R., Moore, K. P., Mani, A. R. 2012. Role of endogenous hydrogen sulfide in neurogenic relaxation of rat corpus cavernosum. Biochemical Pharmacology. 83(9). 1261-68.

Glazer, A. N., McKenzie, H. A., Wake, R. G. 1963. The denaturation of proteins: II. Ultraviolet absorption spectra of bovine serum albumin and ovalbumin in urea and in acid solution. Biochimica et Biophysica Acta. 69. 240-48.

Haccard, O., Lewellyn, A., Hartley, R. S., Erikson, E., Maller, J. L. 1995. Induction of Xenopus oocyte meiotic maturation by MAP kinase. Developmental Biology. 168(2). 677-82.

Han, Z. B., Lan, G. C., Wu, Y. G., Han, D., Feng, W. G., Wang, J. Z., Tan, J. H. 2006. Interactive effects of granulosa cell apoptosis, follicle size, cumulus–oocyte complex morphology, and cumulus expansion on the developmental competence of goat oocytes: a study using the well-in-drop culture system. Reproduction. 132(5). 749-58.

Harada, T., Koi, H., Kubota, T., Aso, T. 2004. Haem oxygenase augments porcine granulosa cell apoptosis in vitro. Journal of Endokrinology. 181(1). 191-205.

Hirshfield, A. N. 1991. Theca cells may be present at the outset of follucular growth. Biology of Reproduction. 44(6). 1157-162.

Hu, R., Lu, J., You, X., Zhu, X., Hui, N., Ni, X. 2011. Hydrogen sulfide inhibits the spontaneous and oxytocin-induced contractility of human pregnant myometrium. Gynecological Endocrinology. 27(11). 900-904.

Hu, Y., Chen, X., Pan, T. T., Neo, K. L., Lee, S. W., Khin, E. S. W., Bian, J. S. 2008. Cardioprotection induced by hydrogen sulfide preconditioning involves activation of ERK and PI3K/Akt pathways. Pflügers Archiv-European Journal of Physiology. 455(4). 607-16.

Huang, S., Hang, Y. 2010. Simultaneous determination of bisphenol A and tetrabromobisphenol A in plastic products using high performance liquid chromatography-mass spectrometry. Se pu Chinese Journal of Chromatography/Zhongguo hua xue hui. 28(9). 863-66.

Huang, Y., Li, F., Tong, W., Zhang, A., He, Y., Fu, T., Liu, B. 2010. Hydrogen sulfide, a gaseous transmitter, stimulates proliferation of interstitial cells of Cajal via phosphorylation of AKT protein kinase. Tohoku Journal of Experimental Medicine. 221(2).

Hunt, P. A., Koehler, K. E., Susiarjo, M., Hodges, C. A., Ilagan, A., Voigt, R. C., Thomas, S., Hassold, T. J. 2003. Bisphenol A exposure causes meiotic aneuploidy in the female mouse. Current Biology. 13(7). 546-53.

Hunt, P. A., Susiarjo, M., Rubio, C., Hassold, T. J. 2009. The Bisphenol A Experience: A Primer for the Analysis of Environmental Effects on Mammalian Reproduction 1. Biology of Reproduction. 81(5). 807-13.

Hunter, M. G. 2000. Oocyte maturation and ovum quality in pigs. Reviews of Reproduction. 5(2). 122-30.

Hurk, R., Zhao, J. 2005. Formation of mammalian oocytes and their growth, differentiation and maturation within ovarian follicles. Theriogenology. 63(6). 1717-51.

Chen, H., Kui, C., Chan, H. C. 2013. Ca^{2+} mobilization in cumulus cells: role in oocyte maturation and acrosome reaction. Cell Calcium. 53(1). 68-75.

Chen, L. Y., Chen, Q., Zhu, X. J., Kong, D. S., Wu, L., Shao, J. J., Zheng, S. Z. 2016. Diallyl trisulfide protects against ethanol-induced oxidative stress and apoptosis via a hydrogen sulfide-mediated mechanism. International Immunopharmacology. 36. 23-30.

Chen, L., Mao, S. J., Larsen, W. J. 1992. Identification of a factor in fetal bovine serum that stabilizes the cumulus extracellular matrix. A role for a member of the inter-alpha-trypsin inhibitor family. Journal of Biological Chemistry. 267(17). 12380-86.

Chen, L., Russell, P. T., Larsen, W. J. 1993. Functional significance of cumulus expansion in the mouse: roles for the preovulatory synthesis of hyaluronic acid within the cumulus mass. Molecular Reproduction and Development. 34(1). 87-93.

Chen, L., Wert, S. E., Hendrix, E. M., Russell, P. T., Cannon, M., Larsen, W. J. 1990. Hyaluronic acid synthesis and gap junction endocytosis are necessary for normal expansion of the cumulus mass. Molecular Reproduction and Development. 26(3). 236-47.

Chen, Q., Li, X. L., Liu, Q., Jiano, Q. C., Cao, W. G., Wan, H. 2005. Investigation the binding interaction of azur A with hyaluronic acid via spectrophotometry and its analytical application. Analytical and Bioanalytical Chemistry. 382(7). 1513-519.

Chmelíková, E., Ješeta, M., Sedmíková, M., Petr, J., Tůmová, L., Kott, T., Lipovová, P., Jílek, F. 2010. Nitric oxide synthase isoforms and the effect of their inhibition on meiotic maturation of porcine oocytes. Zygote. 18(03). 235-44.

Chuah, S. C., Moore, P. K., Zhu, Y. Z. 2007. S-allylcysteine mediates cardioprotection in anacute myocardial infarction rat model via a hydrogen sulfide-mediated pathway. AmericanJournal of Physiology - Heart and Circulatory Physiology. 293(5). H2693-701.

Iwakiri, Y. 2011. S-nitrosylation of proteins: a new insight into endothelial cell function regulated by eNOS-derived NO. Nitric Oxide. 25(2). 95-101.

Jablonka-Shariff, A., Olson, L. M. 1998. The Role of Nitric Oxide in Oocyte Meiotic Maturation and Ovulation: Meiotic Abnormalities of Endothelial Nitric Oxide Synthase Knock-Out Mouse Oocytes 1. Endocrinology. 139(6). 2944-54.

Jablonka-Shariff, A., Olson, L. M. 2000. Nitric oxide is essential for optimal meiotic maturation of murine cumulus-oocyte complexes in vitro. Molecular Reproduction and Development. 55(4). 412-21.

Jablonka-Shariff, A., Ravi, S., Beltsos, A. N., Murphy, L. L., Olson, L. M. 1999. Abnormal estrous cyclicity after disruption of endothelial and inducible nitric oxide synthase in mice. Biology of Reproduction. 61(1). 171-77.

Ješeta, M., Marin, M., Tichovská, H., Melicharová, P., Cailliau-Maggio, K., Martoriati, A., Lescuyer-Rousseau, A., Beaujois, R., Petr, J., Sedmíková, M., Bodart, J. F. 2012. Nitric oxide-donor SNAP induces Xenopus eggs activation. PloS One. 7(7). e41509.

Ježová, M., Scsuková, S., Nagyová, E., Vranová, J., Procházka, R., Kolena, J. 2001. Effect of intraovarian factors on porcine follicular cells: cumulus expansion, granulosa and cumulus cell progesterone production. Animal Reproduction Science. 65(1-2). 115-26.

Ji, K., Hong, S., Kho, Y., Choi, K. 2013. Effects of bisphenol S exposure on endocrine functions and reproduction of zebrafish. Environmental Science & Technology. 47(15). 8793-800.

Johnson, J., Bagley, J., Skaznik–Wikiel, M., Lee, H. J., Adams, G. B., Niikura, Y., Tschudy, K. S., Tilly, J. C., Cortes, M. L., Forkert, R., Spitzer, T., Iacomini, J., Scadden, D. T., Tilly, J. L. 2005. Oocyte generation in adult mammalian ovaries by putative germ cells in bone marrow and peripheral blood. Cell. 122(2). 303–15.

Johnson, J., Canning, J., Kaneko, T., Pru, J. K., Tilly, J. L. 2004. Germline stem cells and follicular renewal in the postnatal mammalian ovary. Nature. 428(6979). 145-50.

Jones, K. T. 2004. Turning it on and off: M-phase promoting factor during meiotic maturation and fertilization. Molecular Human Reproduction. 10(1). 1-5.

Ju, S., Rui, R. 2012. Effects of cumulus cells on in vitro maturation of oocytes and development of cloned embryos in the pig. Reproduction in Domestic Animals, 47(4), 521-29.

Kalous, J., Kubelka, M., Šolc, P., Šušor, A., Motlík, J. 2009. AKT (protein kinase B) is implicated in meiotic maturation of porcine oocytes. Reproduction. 138(4). 645-54.

Karja, N. W. K. 2008. Nuclear maturation of porcine oocytes in vitro: effect of the cumulus-oocyte complexes quality. Indonesian Journal of Biotechnology. 13(2). 1078-84.

Kim, A. M., Bernhardt, M. L., Kong, B. Y., Ahn, R. W., Vogt, S., Woodruff, T. K., O´Halloran, T. V. 2011. Zinc sparks are triggered by fertilization and facilitace cell cycle resumption in mammalian eggs. ACS Chemical Biology. 6(7). 716-23.

Kim, A. M., Vogt, S., O'Halloran, T. V., Woodruff, T. K. 2010. Zinc availability regulates exit from meiosis in maturing mammalian oocytes. Nature Chemical Biology. 6(9). 674-81.

Kimura, N., Konno, Y., Miyoshi, K., Matsumoto, H., Sato, E. 2002. Expression of hyaluronan synthases and CD44 messenger RNAs in porcine cumulus-oocyte complexes during in vitro maturation. Biology of reproduction. 66(3). 707-17.

Kishimoto, T. 2003. Cell-cycle control during meiotic maturation. Current Opinion in Cell Biology. 15(6). 654-63.

Kong, B. Y., Bernhardt, M. L., Kim, A. M., O'Halloran, T. V., Woodruff, T. K. 2012. Zinc maintains prophase I arrest in mouse oocytes through regulation of the MOS-MAPK pathway. Biology of Reproduction. 87(1). 1-12.

Kongtawelert, P., Ghosh, P. 1990. A method for the quantification of hyaluronan (hyaluronic acid) in biological fluids using a labeled avidin-biotin technique. Analytical Biochemistry. 185(2). 313-18.

Kreiser, D., Kelly, D. K., Seidman, D. S., Stevenson, D. K., Baum, M., Dennery, P. A. 2003. Gestational pattern of heme oxygenase expression in the rat. Pediatric Research. 54(2). 172-78.

Krüger, T., Long, M., Bonefeld-Jørgensen, E. C. 2008. Plastic components affect the activation of the aryl hydrocarbon and the androgen receptor. Toxicology. 246(2). 112-23.

Kubelka, M., Motlík, J., Schultz, R. M., Pavlok, A. 2000. Butyrolactone I Reversibly Inhibits Meiotic Maturation of Bovine Oocytes, Without Influencing Chromosome Condensation Activity 1. Biology of Reproduction. 62(2). 292-302.

Laforest, M. F., Pouliot, É., Guéguen, L., Richard, F. J. 2005. Fundamental significance of specific phosphodiesterases in the control of spontaneous meiotic resumption in porcine oocytes. Molecular Reproduction and Development. 70(3). 361-72.

Lamas, S., Marsden, P. A., Li, G. K., Tempst, P., Michel, T. 1992. Endothelial nitric oxide synthase: molecular cloning and characterization of a distinct constitutive enzyme isoform. Proceedings of the National Academy of Sciences. 89(14). 6348-52.

Li, T. Y., Colley, D., Barr, K. J., Yee, S. P., Kidder, G. M. 2007. Rescue of oogenesis in Cx37-null mutant mice by oocyte-specific replacement with Cx43. Journal of Cell Science. 120(23). 4117-25.

Liang, C. G., Huo, L. J., Zhong, Z. S., Chen, D. Y., Schatten, H., Sun, Q. Y. 2005. Cyclic adenosine 3′,5′-monophosphate-dependent activation of mitogen-activated protein kinase in cumulus cells is essential for germinal vesicle breakdown of porcine cumulusenclosed oocytes. Endocrinology. 146 (10). 4437-44.

Liang, C. H., Su, Y., Fan, H., Schatten, H., Sun, Q. 2007. Mechanisms regulating oocyte meiotic resupmtion: roles of mitogen-activated protein kinase. Molecular Endokrinology. 21(9). 2037-55.

Liang, R., Yu, W. D., Du, J. B., Yang, L. J., Shang, M., Guo, J. Z. 2006. Localization of cystathionine beta synthase in mice ovaries and its expression profile during follicular development. Chinese Medical Journal. 119(22). 1877-83.

Liang, R., Yu, W. D., Du, J. B., Yang, L. J., Yang, J. J., Jian, X., Shang, M., Guo, J. Z. 2007: Cystathionine β synthase participates in murine oocytes maturatione mediated by homocysteine. Reproductive Toxicology. 24(1). 89-96.

Liao, C., Liu, F., Kannan, K. 2012. Bisphenol S, a new bisphenol analogue, in paper products and currency bills and its association with bisphenol A residues. Environmental Science & Technology. 46(12). 6515-22.

Lisle, R. S., Anthony, K., Randall, M. A., Diaz, F. J. 2013. Oocyte-cumulus cell interactions regulate free intracellular zinc in mouse oocytes. Reproduction. 145(4). 381-90.

Liu, Z., de Matos, D. G., Fan, H. Y., Shimada, M., Palmer, S., Richards, J. S. 2009. Interleukin-6: an autocrine regulator of the mouse cumulus cell-oocyte complex expansion process. Endocrinology. 150(7). 3360-68.

Lodish, H., Berk, A., Matsudaira, P., Kaiser, C. A., Krieger, M., Scott, M. P., Zipursky, L., Darnell, J. 2004. Molecular Cell Biology 5th. edition. Palgrave Macmillan, W. H. Freeman. p. 967. ISBN: 9780716743668.

Louis, X. L., Murphy, R., Thandapilly, S. J., Yu, L., Netticadan, T. 2012. Garlic extracts prevent oxidative stress, hypertrophy and apoptosis in cardiomyocytes: a role for nitric oxide and hydrogen sulfide. BMC Complementary and Alternative Medicine. 12(1). 140.

Lugnier, C. 2006. Cyclic nucleotide phosphodiesterase (PDE) superfamily: a new target for the development of specific therapeutic agents. Pharmacology & Therapeutics. 109(3). 366-98.

Maines, M. D. 1988. Heme oxygenase: function, multiplicity, regulatory mechanisms, and clinical applications. The FASEB Journal. 2(10). 2557-68.

Markey, C. M., Wadia, P. R., Rubin, B. S., Sonnenschein, C., Soto, A. M. 2005. Long-term effects of fetal exposure to low doses of the xenoestrogen Bisphenol-A in the female mouse genital tract 1. Biology of Reproduction. 72(6). 1344-51.

Mattioli, M., Gioia, L., Barboni, B. 1998. Calcium elevation in Wheel cumulus-oocyte complexes after luteinising hormone stimulation. Molecular Reproduction and Development. 50(3). 361-69.

McCann, S. M. 1982. Physiology and pharmacology of LHRH and somatostatin. Annual Review of Pharmacology and Toxikology. 22(1). 491-515.

McCoubrey, W. K., Huang, T. J., Maines, M. D. 1997. Isolation and characterization of a cDNA from the rat brain that encodes hemoprotein heme oxygenase-3. European Journal of Biochemistry. 247(2). 725-32.

Mehlmann, L. M. 2005. Stops and starts in mammalian oocyte: recent advances in understanding the regulation of meiotic arrest and oocyte maturation. Reproduction. 130(6). 791-99.

Miething, H. 1988. HPLC-analysis of the volatile oil of garlic bulbs. Phytotherapy Research. 2(3). 149-51.

Michałowicz, J., Mokra, K., Bąk, A. 2015. Bisphenol A and its analogs induce morphological and biochemical alterations in human peripheral blood mononuclear cells (in vitro study). Toxicology in Vitro. 29(7). 1464-72.

Mlynarčíková, A., Nagyová, E., Ficková, M., Scsuková, S. 2009. Effects of selected endocrine disruptors on meiotic maturation, cumulus expansion, synthesis of hyaluronan and progesterone by porcine oocyte-cumulus complexes. Toxicology In Vitro. 23(3). 371-77.

Moor, R. M., Mattioli, M., Ding, J., Nagai, T. 1990. Maturation of pig oocytes *in vivo* and *in vitro*. Journal of Reproduction and Fertility. (Supplement No. 40). 197-210.

Motlík, J., Crozet, N., Fulka, J. 1984. Meiotic competence in vitro of pig oocytes isolated from early antral follicles. Journal of Reproduction and Fertility. 72(2). 323-28.

Motlík, J., Fulka, J. 1976. Breakdown of germinal vesicle in pig oocytes in vivo and in vitro. Journal of Experimental Zoology. 198(2). 155–62.

Motlík, J., Procházka, R., Nagyová, E., Schellander, K., Brem, G. 1998. Paracrine and autocrine regulation of cumulus expansion in porcine folllicles. Reproduction in Domestic Animals. 33(3-4). 181-85.

Motta, P. M., Nottola, S. A., Makabe, S. 1997. Natural history of the female germ cell from its origin to full maturation through prenatal ovarian development. European Journal of Obstetrics and Gynecology and Reproductive Biology. 75(1). 5-10.

Mustafa, A. K., Gadalla, M. M., Sen, N., Kim, S., Mu, W., Gazi, S. K., Snyder, S. H. 2009. H_2S signals through protein S-sulfhydration. Science Signaling. 2(96). 72.

Mustafa, A. K., Gadalla, M. M., Snyder, S. H. 2009. Signaling by gasotransmitters. Science Signaling, 2(68), re2.

Nagyová E, Vanderhyden B, Procházka R (2000): Secretion of paracrine factors enabling expansion of cumulus cells is developmentally regulated in pig oocytes 1. Biology of Reproduction. 63(4). 1149-56.

Nagyová, E., Camaioni, A., Prochazka, R., Salustri, A. 2004. Covalent transfer of heavy chains of inter-alpha-trypsin inhibitor fymily proteins to hyaluronan *in vivo* and *in vitro* expanded porcine oocyte-cumulus complexes. Biology of Reproduction. 71(6). 1838-43.

Nagyová, E., Procházka, R., Vanderhyden, B. C. 1999. Oocytectomy does not influence synthesis of hyaluronic acid by pig cumulus cells: retention of hyaluronic acid after insulin-like growth factor-I treatment in serum-free medium. Biology of Reproduction. 61(3). 569-74.

Nakayama, T., Inoue, M., Sato, E. 1996. Effect of oocytectomy on glycosaminoglycan composition during cumulus expansion of porcine cumulus-oocyte complexes cultured in vitro. Biology of Reproduction. 55(6). 1299-1304.

Němcová, L., Nagyová, E., Petlach, M., Tománek, M., Procházka, R. 2007. Molecular Mechanisms of Insulin-Like Growth Factor 1 Promoted Synthesis and Retention of Hyaluronic Acid in Porcine Oocyte-Cumulus Complexes. Biology of Reroduction. 76(6). 1016-24.

Nevoral, J., Petr, J., Gelaude, A., Bodart, J. F., Kučerová-Chrpová, V., Sedmikova, M., Krejčová, T., Kolbabová, T., Dvořáková, M., Vyskočilová, A., Weingartová, I., Křivohlávková, L., Žalmanová, T., Jílek, F. 2014. Dual effects of hydrogen sulfide donor on meiosis and cumulus expansion of porcine cumulus-oocyte complexes. PloS One. 9(7). e99613.

Ning, N., Zhu, J., Du, Y., Gao, X., Liu, C., Li, J. 2014. Dysregulation of hydrogen sulphide metabolism impairs oviductal transport of embryos. Nature Communications, 5.

Njie-Mbye, Y. F., Kulkarni, M., Opere, C. A., Ohia, S. E. 2012. Mechanism of action of hydrogen sulfide on cyclic AMP formation in rat retinal pigment epithelial cells. Experimental Eye Research. 98. 16-22.

Olson, K. R., Donald, J. A., Dombkowski, R. A., Perry, S. F. 2012. Evolutionary and comparative aspects of nitric oxide, carbon monoxide and hydrogen sulfide. Respiratory Physiology & Neurobiology. 184(2). 117-29.

Palmer, A., Gavin, A. C., Nebreda, A. R. 1998. A link between MAP kinase and p34(cdc2)/cyclin B during oocyte maturation: p90(rsk) phosphorylates and inactivates the p34(cdc2) inhibitory kinase Myt1. The EMBO Journal. 17 (17). 5037-47.

Parikh, F. R., Nadkarni, S. G., Naik, N. J., Naik, D. J., Uttamchandani, S. A. 2006. Cumulus coculture and cumulus-aided embryo transfer increases pregnancy rates in patients undergoing in vitro fertilization. Fertility and Sterility. 86(4). 839-47.

Patel, P., Vatish, M., Heptinstall, J., Wang, R., Carson, R. J. 2009. The endogenous production of hydrogen sulphide in intrauterine tissues. Reproductive Biology and Endocrinology. 7(1). 10.

Patisaul, H. B., Fortino, A. E., Polston, E. K. 2006. Neonatal genistein or bisphenol-A exposure alters sexual differentiation of the AVPV. Neurotoxicology and Theratology. 28(1). 111-18.

Petr, J., Chmelíková, E., Krejčová, T., Řehák, D., Novotná, B., Jílek, F. 2010. Parthenogenetic activation of pig oocytes using pulsatile treatment with a nitric oxide donor. Reproduction in Domestic Animals. 45(3). 493-99.

Petr, J., Rajmon, R., Rozinek, J., Sedmíková, M., Ješeta, M., Chmelíková, E., Švestková, D., Jílek, F. 2005. Activation of pig oocytes using nitric oxide donors. Molecular Reproduction and Development. 71(1). 115-22.

Petr, J., Urbánková, D., Tománek, M., Rozinek, J., Jílek, F. 2002. Activation of in vitro matured pig oocytes using activators of inositol triphosphate or ryanodine receptors. Animal Reproduction Science. 70 (3). 235-49.

Prasad, K., Laxdal, V. A., Yu, M., Raney, B. L. 1996. Evaluation of hydroxyl radical-scavenging property of garlic. Molecular and Cellular Biochemistry. 154(1). 55-63.

Procházka, R., Nagyová, E., Brem, G., Schellander, K., Motlík, J. 1998. Secretion of cumulus expansion-enabling factor (CEEF) in porcine follicles. Molecular Reproduction and Development. 49.(2). 141-49.

Procházka, R., Sršeň, V., Nagyová, E., Miyano, T., & Flechon, J. E. 2000. Developmental regulation of effect of epidermal growth factor on porcine oocyte–cumulus cell complexes: Nuclear maturation, expansion, and F-actin remodeling. Molecular Reproduction and Development. 56(1). 63-73.

Qian, Y., Shi, W. Q., Ding; J. T., Sha, J. H., Fan, B. Q. 2003. Predictive value of the area of expanded cumulus mass on development of porcine oocytes matured and fertilized in vitro. Journal of Reproduction and Development. 49 (2). 167-74.

Ramos, J. G., Varayoud, J., Kass, L., Rodríguez, H., Costabel, L., Muñoz-de-Toro, M., Luque, E. H. 2003. Bisphenol A induces both transient and permanent histofunctional alterations of the hypothalamic-pituitary-gonadal axis in prenatally exposed male rats. Endocrinology. 144(7). 3206-15.

Reich, R., Miskin, R., Tsafriri, A. 1985. Follicular plasminogen activator: involvement in ovulation. Endocrinology. 116 (2). 516-21.

Richards, J. S., Russell, D. L., Ochsner, S., Hsieh, M., Doyle, K. H., Falender, A. E., Lo, Y. K., Sharma, S. C. 2002. Novel signalling pathways that control ovarian follicular development, ovulation, and luteinization. Recent Progress in Hormon Research. 57(1). 195-220.

Robinson, J. W., Zhang, M., Shuhaibar, L. C., Norris, R. P., Geerts, A., Wunder, F., Eppig, J. J., Potter, L.R., Jaffe, L. A. 2012. Luteinizing hormone reduces the activity of the NPR2 guanylyl cyclase in mouse ovarian follicles, contributing to the cyclic GMP decrease that promotes resumption of meiosis in oocytes. Developmental Biology. 366(2). 308-16.

Rosen, M. P., Zamah, A. M., Shen, S., Dobson, A. T., McCulloch, C. E., Rinaudo, P. F., Lamb, J. D., Cedars, M. I. 2009. The effect of follicular fluid hormones on oocyte recovery after ovarian stimulation: FSH level predicts oocyte recovery. Reproductive Biology and Endocrinology. 7(1). 35.

Rozinek, J., Petr, J., Antalíková, L. 1991. Morphology of cortical parts in growing and fully-grown pig oocytes. XXXIIIrd Congres of Czechoslovak Anatomical Society.

Rugg, M. S., Willis, A. C., Mukhopadhyay, D., Hascall, V. C., Fries, E., Fülöp, C., Milner, C.M., Day, A. J. 2005. Characterization of complexes formed between TSG-6 and inter-α-inhibitor that act as intermediates in the covalent transfer of heavy chains onto hyaluronan. Journal of Biological Chemistry. 280(27). 25674-86.

Russell, D. L., Salustri, A. 2006. Extracellular matrix of the cumulus-oocyte complex. Seminars in Reproductive Medicine. 24(4). 217-27.

Sadler, T. W. 2011. Langmanova lékařská embryologie. Grada Publishing, a. s. p. 414. ISBN: 8024726408.

Saitoh, H., Takagaki, K., Majima, M., Nakamura, T., Matsuki, A., Kasai, M., Endo, M. 1995. Enzymic reconstruction of glycosaminoglycan oligosaccharide chains using the transglycosylation reaction of bovine testicular hyaluronidase. Journal of Biological Chemistry. 270(8). 3741-47.

Salustri, A. 2000. Paracrine actions of oocytes in the mouse pre-ovulatory follicle. International Journal of Developmental Biology. 44(6). 591-97.

Salustri, A., Ulisse, S., Yanagishita, M., Hascall, V. C. 1990. Hyaluronic acid synthesis by mural granulosa cells and cumulus cells in vitro is selectively stimulated by a factor produced by oocytes and by transforming growth factor-β. The Journal of Biological Chemistry. 265(32). 19517–23.

Salustri, A., Yanagishita, M., Hascall, V. C. 1989. Synthesis and accumulation of hyaluronic acid and proteoglycans in the mouse cumulus cell-oocyte complex during follicle-stimulating hormone-induced mucification. Journal of Biological Chemistry. 264(23). 13840-47.

Sasseville, M., Côté, N., Gagnon, M. C., Richard, F. J. 2008. Up-regulation of 3′ 5′-cyclic guanosine monophosphate-specific phosphodiesterase in the porcine cumulus-oocyte complex affects steroidogenesis during in vitro maturation. Endocrinology. 149(11). 5568-76.

Scarchilli, L., Camaioni, A., Bottazzi, B., Negri, V., Doni, A., Deban, L., Bastone, a., Salvatori, G., Mantovani, A., Siracusa, G., Salustri, A. 2007. PTX3 interacts with inter-α-trypsin inhibitor implications for hyaluronan organization and cumulus oophorus expansion. Journal of Biological Chemistry. 282(41). 30161-70.

Shibuya, N., Tanaka, M., Yoshida, M., Ogasa-Wara, Y., Togawa, T., Ishii, K., Kimura, H. 2009. 3-mercaptopyruvate sulfurtransferase produces hydrogen sulfide and bound sulfane sulfur in the brain. Antioxidants and Redox Signaling. 11(4). 703-14.

Schwarz, K. R., Pires, P. R., Adona, P. R., de Bem, T. H. C., Leal, C. L. 2008. Influence of nitric oxide during maturation on bovine oocyte meiosis and embryo development in vitro. Reproduction, Fertility and Development. 20(4). 529-36.

Sidhu, R., Singh, M., Samir, G., Carson, R. J. 2001. L-cysteine and sodium hydrosulphide inhibit spontaneous contractility in isolated pregnant rat uterine strips in vitro. Basic & Clinical Pharmacology & Toxicology. 88(4). 198-203.

Sirard, M. A., Dubuc, A., Bolamba, D., Zheng, Y., Coenen, K. 1993. Follicle-oocyte-sperm interactions in vitro and in vitro in pigs. Journal of Reproduction and Fertility. Supplement 48. 3-16.

Solursh, M. 1976. Glycosaminoglycan synthesis in the chick gastrula. Developmental Biology. 50(2). 525-30.

Stern, R., Jedrzejas, M. J. 2006. Hyaluronidases: their genomics, structures and mechanisms of action. Chemical Reviews. 106(3). 818-39

Strickland, S., Beers, W. H. 1976. Studies on the role of plasminogen activator in ovulation. *In vitro* response of granulosa cells to gonadotropins, cyclic nucleotides, and prostaglandins. The Journal of Biological Chemistry. 251(18). 5694-702.

Sugiura, K., Su, Y. Q., Li, Q., Wigglesworth, K., Matzuk, M. M., Eppig, J. J. 2010. Estrogen promotes the development of mouse cumulus cells in coordination with oocyte-derived GDF9 and BMP15. Molecular Endocrinology. 24(12). 2303-14.

Sugiura, Y., Kashiba, M., Maruyama, K., Hoshikawa, K., Sasaki, R., Saito, K., Kimura, H., Goda, N., Suematsu, M. 2005. Cadmium exposure alters metabolomics of sulfur-containing amino acids in rat testes. Antioxidants & Redox Signaling. 7(5-6). 781-87.

Sun, Q. Y., Miao, Y. L., Schatten, H. 2009. Towards a new understanding on the regulation of mammalian oocyte meiosis resumption. Cell Cycle. 8(17). 2741-7.

Sun, Q., Nagai, T. 2003. Molecular mechanisms underlying pig oocyte maturation and fertilization. Journals of Reproduction and Development. 49(5). 347-59.

Sun, Y. G., Wang, X. Y., Chen, X., Shen, C. X., Li, Y. G. 2015. Hydrogen sulfide improvescardiomyocytes electrical remodeling post ischemia/reperfusion injury in rats. International Journal of Clinical and Experimental Pathology. 8(1). 474-81.

Šutovský, P., Fléchon, J. E., Pavlok, A. 1994. Microfilaments, microtubules and intermediate filaments fulfil differential roles during gonadotropin-induced expansion of bovine cumulus oophorus. Reproduction Nutrition Development. 34(5). 415-25.

Takagaki, K., Takeda, Y., Nakamura, T., Daidouji, K., Narita, H., Endo, M. 1994. Analysis of glycosaminoglycans by high-performance liquid chromatography. Journal of Biochemical and Biophysical Methods. 28(4). 313-20.

Tamba, S., Yodoi, R., Segi-Nishida, E., Ichikawa, A., Narumiya, S., Sugimoto, Y. 2008. Timely interaction between prostaglandin and chemokine signaling is a prerequisite for successful fertilization. Proceedings of the National Academy of Sciences. 105(38). 14539-44.

Tang, G., Wu, L., Wang, R. 2010. Interaction of hydrogen sulfide with ion channels. Clinical and Experimental Pharmacology and Physiology. 37(7). 753-63.

Tanghe, S., van Soom, A., Nauwynck, H., Coryn, M., de Kruif, A. 2002. Minireview: Functions of the cumulus oophorus during oocyte maturation, ovulation, and fertilization. Molecular Reproduction and Development. 61(3). 414-24.

Tao, Y., Xie, H., Hong, H., Chen, X., Jang, J., Xia, G. 2005. Effects of nitric oxide synthase inhibitors on porcine oocyte meiotic maturation. Zygote. 13(01). 1-9.

Tatemoto, H., Sakurai, N., Muto, N. 2000. Protection of porcine oocytes against apoptotic cell death caused by oxidative stress during *in vitro* maturation: role of cumulus cells. Biology of Reproduction. 63(3). 805-10.

Tirone, E., Siracusa, G., Hascall, V. C., Frajese, G., Salustri, A. 1993. Oocytes preserve the ability of mouse cumulus cells in culture to synthesize hyaluronic acid and dermatan sulfate. Developmental Biology. 160(2). 405-12.

Trakshel, G. M., Kutty, R. K., Maines, M. D. 1986. Purification and characterization of the major constitutive form of testicular heme oxygenase. The noninducible isoform. Journal of Biological Chemistry. 261(24). 11131-37.

Tsai, C. Y., Wen, S. Y., Shibu, M. A., Yang, Y. C., Peng, H., Wang, B., Wei, Y. M., Chang, H. Y., Lee, C. Y., Kuo, W. W. 2015. Diallyl trisulfide protects against high glucose-induced cardiac apoptosis by stimulating the production of cystathionine gamma-lyase-derived hydrogen sulfide. International Journal of Cardiology. 195. 300-10.

Ueno, S., Yoshida, N., Niimura, S. 2009. Amount of hyaluronan produced by mouse oocytes and role of hyaluronan in enlargement of the perivitelline space. Journal of Reproduction and Development, 55(5). 496-501.

Vabres, P. 2010: Hyaluronan, embryogenesis and morpho-genesis. Annales de Dermatologie et de Vénéréologie. 137. S9-S14.

Van Voorhis, B. J., Moore, K., Strijbos, P. J., Nelson, S., Baylis, S. A., Grzybicki, D., Weiner, C. P. 1995. Expression and localization of inducible and endothelial nitric oxide synthase in the rat ovary. Effects of gonadotropin stimulation in vivo. Journal of Clinical Investigation. 96(6). 2719.

Vandenberg, L. N., Maffini, M. V., Wadia, P. R., Sonnenschein, C., Rubin, B. S., Soto, A. M. 2007. Exposure to environmentally relevant doses of the xenoestrogen bisphenol-A alters development of the fetal mouse mammary gland. Endocrinology. 148(1). 116-27.

Vanderhyden, B. C. 1993. Species differences in the regulation of cumulus expansion by an oocyte-secreted factor(s). Journal of Reproduction and Fertility. 98(1). 219-27.

Vanderhyden, B. C., Caron, P. J., Buccione, R., Eppig, J. J. 1990. Developmental pattern of the secretion of cumulus expansion-enabling factor by mouse oocytes and the role of oocytes in promoting granulosa cell differentiation. Developmental Biology. 140(2). 307-17.

Vanderhyden, B. C., Macdonald, E. A., Nagyova, E., Dhawan, A. 2003. Evaluation of members of the TGFß superfamily as candidates for the oocyte factors that control mouse cumulus expansion and steroidogenesis. Reprod Suppl. 61. 55-70.

Vinas, R., Watson, C. S. 2013. Bisphenol S disrupts estradiol-induced nongenomic signaling in a rat pituitary cell line: effects on cell functions. Environmental Health Perspectives. 121(3). 352.

Volpi, N. 2000. Hyaluronic acid and chondroitin sulfate unsaturated disacharides analysis by High-Performance Liquis Chromatography and fluorometric detection with dansylhydrazine. Analytical Biochemistry. 277(1). 19-24.

Wang, R. 2002. Two's company, three'sa crowd: can H_2S be the third endogenous gaseous transmitter?. The Faseb Journal. 16(13). 1792-98.

Wang, T., Han, J., Duan, X., Xiong, B., Cui, X. S., Kim, N. H., Liu, H. L., Sun, S. C. 2016. The toxic effects and possible mechanisms of Bisphenol A on oocyte maturation of porcine in vitro. Oncotarget. 7(22). 32554-65.

Wang, Y., Liang, N., Yao, G., Tian, H., Zhai, Y., Yin, Y., Sun, F. 2014. Knockdown of TrkA in cumulus oocyte complexes (COCs) inhibits EGF-induced cumulus expansion by down-regulation of IL-6. Molecular and Cellular Endocrinology. 382(2). 804-13.

Wang., W., Sun, Q., Hosoe, M., Shioya, Y., Day, B. N. 1997. Quantified analysis of cortical granule distribution and exocytosis of porcine oocytes during meiotic maturation and activation. Biology of Reproduction. 56(6). 1376-82.

Wassarman, P. M. 1988. The Mammalian Ovum In: The Physiology of Reproduction. eds. Knobil E. a Neill J., Raven Press Ltd., New York. 69-102.

Wassarman, P. M., Albertini, D. F. 1994. The Mammalian Ovum, Second Edition. The Physiology of Reproduction. Raven Press. New York. 79-122.

Watson, C. S., Bulayeva, N. N., Wozniak, A. L., Finnerty, C. C. 2005. Signaling from the membrane via membrane estrogen receptor-α: estrogens, xenoestrogens, and phytoestrogens. Steroids. 70(5). 364-71.

Ye, J., Flint, A. P. F., Luck, M. R., Campbell, K. H. S. 2003. Independent activation of MAP kinase and MPF during the initiation of meiotic maturation in pig oocytes. Reproduction. 125(5). 645-56.

Yokoo, M., Kimura, N., Sato, E. 2010. Induction of oocyte maturation by hyaluronan-CD44 interaction in pigs. Journals of Reproduction and Development. 56 (1). 15-19.

Yokoo, M., Miyahayashi, Y., Naganuma, T., Kimura, N., Sasada, H., Sato, E. 2002. Identification of hyaluronic acid-binding proteins and their expressions in porcine cumulus-oocyte complexes during *in vitro* maturation. Biology of Reproduction. 67(4). 1165-71.

Yokoo, M., Nagahara, T., Kimura, N., Shimizu, T., Matsumoto, H., Sasada, H., Sato, E. 2003- Hyaluronan-CD44 system during cumulus expansion induces oocyte maturation in pig. Biology of Reproduction. 68. 325-26.

Yokoo, M., Shimizu, T., Kimura, N., Tunjung, W. A. S., Matsumoto, H., Abe, H., Sasada, H., Rodriguez-Martinez, H., Sato, E. 2007. Role of the hyaluronan receptor CD44 during porcine oocyte maturation. Journal of Reproduction and Development. 53(2). 263-70.

Yokoo, M., Shimizu, T., Kimura, N., Tunjung, W., Matsumoto, H., Abe, H., Sasada, H., Rodriguez-Martinez, H., Sato, E. 2007. Role of the hyaluronan CD44 during porcine oocyte maturation. Journals of Reproduction and Development. 53(2). 263-70.

Yosizawa, Z., Ototani, N., Satake, S. 1983. A simple method for the quantitation of glycuronic acid-containing glycosaminoglycans with mucopolysaccharidases. Analytical Biochemistry. 128(1). 250-56.

Zenclussen, M. L., Jensen, F., Rebelo, S., El-Mousleh, T., Casalis, P. A., Zenclussen, A. C. 2012. Heme oxygenase-1 expression in the ovary dictates a proper oocyte ovulation, fertilization, and corpora lutea maintenance. American Journal of Reproductive Immunology. 67(5). 376-82.

Zhang, L., Underhill, C. B., Chen, L. 1995. Hyaluronan on the surface of tumor cells is correlated with metastatic behavior. Cancer Research. 55(2). 428-433.

Zhang, X., Han, Y. B., Sui, H. S., Miao, D. Q., Wang, J. Z., Li, K. L., Tan, J. H. 2008. Developmental and hormonal regulation of cumulus expansion and secretion of Cumulus Expansion- Enabling Factor (CEEF) in goat follicles. Molecular Reproduction and Development. 75(9). 1387-95.

Zhuo, L., Yoneda, M., Zhao, M., Yingsung, W., Yoshida, N., Kitagawa, Y., Kawamura, K., Suzuki, T., Kimata, K. 2001. Defect in SHAP-hyaluronan complex causes severe female infertility. A study by inactivation of the bikunin gene in mice. The Journal of Biological Chemismy. 276(11). 7693-96.

Žalmanová, T., Hošková, K., Nevoral, J., Zámostná, K., Petr, J. 2015. Vliv bisfenolu S na meiotické zrání prasečích oocytů, Bilaterálne vedecké symposium Endokrinné disruptory, Bratislava 04.-05.2015, 37.

9 Samostatné přílohy

doi: 10.17221/51/2015-CJAS

A simple method for assessing hyaluronic acid production by cumulus-oocyte complexes

K. Zámostná[1], J. Nevoral[1], T. Kott[2], R. Procházka[3], M. Orsák[4], M. Šulc[4], V. Pajkošová[5], V. Pavlík[6], T. Žalmanová[1], K. Hošková[1], F. Jílek[1], P. Klein[7]

[1]Department of Veterinary Sciences, Faculty of Agrobiology, Food and Natural Resources, Czech University of Life Sciences Prague, Prague, Czech Republic
[2]Institute of Animal Science, Prague-Uhříněves, Czech Republic
[3]Institute of Animal Physiology and Genetics, Liběchov, Czech Republic
[4]Department of Chemistry, Faculty of Agrobiology, Food and Natural Resources, Czech University of Life Sciences Prague, Prague, Czech Republic
[5]Department of Biological and Biochemical Sciences, Faculty of Chemical Technology, University of Pardubice, Pardubice, Czech Republic
[6]Contipro Biotech s.r.o., Dolní Dobrouč, Czech Republic
[7]Biomedical Center, Faculty of Medicine in Pilsen, Charles University in Prague, Pilsen, Czech Republic

ABSTRACT: The cumulus expansion of cumulus-oocyte complex (COC) is an essential regulating process of oocyte maturation and as such it is a possible biomarker of the *in vitro* maturing oocytes quality. Cumulus expansion is usually assessed by non-invasive methods based on visual evaluation with many inaccuracies. On the other hand, analytical measurement of the quantity of hyaluronic acid (HA), the most abundant compound of expanded cumuli, is one of possible methods to evaluate cumulus expansion precisely. Therefore, this study aimed to verify the applicability of HA analysis for evaluating the cumulus expansion and testing oocyte maturation. The COCs were cultured in modified M199 medium for 8–48 h. The samples for the HA analysis were prepared on an 8-hour time scale, and HA retained in COCs was measured using a spectrophotometric method adapted for this purpose. We observed an increasing quantity of HA during the *in vitro* cultivation. A comparison with expanded COCs' classification or expansion area proved the proposed method of HA analysis suitable for the evaluation of cumulus expansion *in vitro*. Our findings consider the quantity of HA-expressed cumulus expansion to be a valuable marker of COC quality enabling an adequate oocyte meiotic stage estimation.

Keywords: oocyte; meiotic maturation; cumulus expansion; glycosaminoglycans; spectrophotometry

INTRODUCTION

A sufficient number of successfully *in vitro* matured oocytes, enclosed in cumulus cells and creating a cumulus-oocyte complex (COC), is key for advances in assisted reproduction. While oocyte maturation occurs, mucification of surrounding cumulus cells, called cumulus expansion, takes place simultaneously. Therefore, cumulus expansion is a significant regulating process of oocyte maturation (summarized in Nevoral et al. 2014).

Gonadotropins and growth factors stimulate cumulus expansion, as well as cumulus expansion enabling factors (CEEFs) incoming from the

Supported by the Czech Ministry of Agriculture (Project No. MZeRO0714 and Project No. NAZV QJ1510138) and by the Czech University of Life Sciences Prague (Project CIGA 20132035).

K. Zámostná and J. Nevoral contributed equally to this work.

doi: 10.17221/51/2015-CJAS

oocyte (Dekel et al. 1979; Prochazka et al. 1998, 2011; Nemcova et al. 2007; Huang and Wells 2010). Cumulus expansion consists of the enlargement of the extracellular matrix based on glycosaminoglycan synthesis, in particular hyaluronic acid (HA) (Nakayama et al. 1996). Cumulus expansion results in the disruption of cumulus cells–oocyte cross-talk and in the suppression of the effect of meiosis inhibiting factors (Yokoo et al. 2007, 2010).

Cumulus expansion intensity and the expression of cumulus expansion markers positively correlate with successful oocyte *in vitro* maturation and subsequent embryonic development (Qian et al. 2003; Flechon et al. 2003; Assou et al. 2010; Davachi et al. 2012). It has been shown that the expression of genes associated with cumulus expansion (*HAS2*, *PTGS2*, *TNFAIP6*, *PTX3*) is a marker of adequate cumulus expansion and thus oocyte quality and its developmental competence (McKenzie et al. 2004; Zhang et al. 2005; Assidi et al. 2010; Yuan et al. 2011). However, simple and less difficult approaches to oocyte quality estimation are more appropriate. As such, general cumulus expansion intensity can be used as the biomarker for the aforementioned changes.

Visual evaluation of expanded COCs (Fagbohun and Downs 1990; Vanderhyden et al. 1990) or their area measurement (Daen et al. 1994) are still used for the evaluation of cumulus expansion (Appeltant et al. 2015; Kubo et al. 2015; Machado et al. 2015). Nevertheless, these methods do not affect the three-dimensional structure of expanded cumuli and cannot uncover differences in HA synthesis after COC treatment. Therefore, the use of radioisotope-labelled HA precursors (Eppig 1980) or analytical assessment of HA by hyaluronidase-induced β-elimination (Volpi 2000) enable to utilize the mentioned advantages. Spectrophotometric analysis of β-eliminated HA measurable at 232 nm (Yosizawa et al. 1983), in particular, offers a simple approach for HA-predicted evaluation of expanded cumuli. Surprisingly, analytical methods of HA analysis are not being used, although more precise results of cumulus expansion can be determined in this way. Based on best knowledge, we hypothesized that introducing the HA analysis of *in vitro* cultured COCs can serve as a predictable marker of cumulus expansion and the corresponding stage of oocyte maturation.

Our results indicate that the spectrophotometric HA analysis is a possible method for the evaluation of cumulus expansion expressed by HA in COCs. The use of a simple analytical method of HA measurement comes out as a suitable tool for oocyte quality prediction by cumulus expansion.

MATERIAL AND METHODS

Chemicals. Unless otherwise stated, all chemicals used for the analytical analysis of HA were purchased from Sigma-Aldrich Co. (St. Louis, USA): phosphate buffered saline, pH 7.4 (PBS; P5368), polyvinyl alcohol (PVA; 341584), hyaluronic acid sodium salt (49775), hyaluronidase from *Streptomyces hyalurolyticus* (SHH, H1136), and bovine testicular hyaluronidase (BTH, H3506).

Analysis of HA by spectrophotometry and High-Performance Liquid Chromatography (HPLC). To determine the HA content, the HA polymer was enzymatically digested by specific hyaluronidase from *Streptomyces hyalurolyticus* (hyaluronan lyase, SHH) (Vanderhyden 1993) with the ability to create β-elimination products. In this reaction, the HA polymer was enzymatically digested to the dimers (HA2) and HA oligomers, creating double bonds. Concurrently, standards of HA tetramers (HA4), hexamers (HA6), octamers (HA8), and decamers (HA10), overall named HA oligomers, were analyzed. These HA oligomers were detected by spectrophotometric measurement in an ultraviolet (UV) absorption spectrum. The solutions were spectrophotometrically measured in Einmalküvetten cuvettes (UV-Küvette mikro, 7592 00, Plastibrand) using Spectronic Helios Gamma UV-Vis spectrophotometer (Thermo Fisher Scientific, Waltham, USA) at 190–280 nm against blank consisting of HA-free adequate enzyme-medium solution.

HPLC was performed simultaneously. The HPLC analysis was performed using an UltiMate 3000 system (Thermo Fisher Scientific) equipped with an autosampler (10°C), column oven (35°C), and PDA detector, monitoring at wavelengths 210 and 235 nm for detection of unsaturated and saturated bonds, respectively, where they show the highest absorption (Dr. M. Hermannová, pers. comm.). The injection volume was 25 µl, mobile phase flow 1.5 ml/min, total run time 40 min. Analyte separation was achieved on a Shodex anion exchange column IEC QA-825 (Shodex, Munich, Germany) using gradient elution. The mobile phase consisted of (a) 0.02M sodium chloride in water, and (b) 0.25M sodium chloride in wa-

doi: 10.17221/51/2015-CJAS

ter. The gradient was as follows: 0 min – 100% A, 25 min – 24% A, 26–32 min – 100% B, re-equilibration 33–40 min – 100% A. A polynomic eight-point calibration curve (from 7 to 1000 µl/ml) for both approaches was used.

In vitro cultivation of COCs and evaluation of oocyte maturation. Porcine ovaries were obtained from non-cycling gilts at the local slaughterhouse (Jatky Plzeň a.s., Czech Republic) and kept at 39°C until arrival at the laboratory. 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) supplemented with 32.5mM sodium bicarbonate, 2.75mM calcium L-lactate, 0.025 mg/ml gentamicin, 6.3mM HEPES, 13.5 IU eCG : 6.6 IU hCG/ml (P.G. 600; Intervet International B.V., Boxmeer, the Netherlands) and 5 % (v/v) foetal bovine serum (Sigma-Aldrich). The COCs were matured for 0–48 h in 4-well Petri dishes (Nunc, Thermo Fisher Scientific) containing 1.0 ml of culture medium, at 39°C in a mixture of 5.0 % CO_2 in air.

At the end of culture, oocytes were denuded by repeated pipetting as described below and 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 their meiotic stage was examined under a phase contrast microscope. Five groups of meiotic maturation stages were determined in accordance with the criteria published by Motlik and Fulka (1976): GV – germinal vesicle, LD – late diakinesis, MI – metaphase I, AITI – anaphase I to telophase I transition, MII – metaphase II.

Classification of expanded cumuli. Cumulus expansion was visually assessed using a subjective scoring system (Vanderhyden et al. 1990). Scores 0 to +4 were attributed to increasing degrees of expansion. Score 0 indicated no observable expansion, +1 minimal observable expansion, +2 extended expansion to several layers, +3 complete expansion excluding *corona radiata*, and +4 indicated complete expansion including *corona radiata*. The data were expressed as percentage portion of COC population.

Area measurement of COCs. For the area measurement, the approach by Daen et al. (1994) was used with slight modifications. In brief, images of the same 25× COCs (25 COCs in group) were prepared using a monochromatic CCD camera ProgRes CT1 (Jenoptik, Jena, Germany) with NIS Elements software (Laboratory Imaging s.r.o., Prague, Czech Republic) on an 8 h time scale. The image analysis based on thresholding and area measurement of 25× COCs including oocyte areas was performed. The data were expressed relative to COCs after 48 h of cultivation.

Expanded cumuli isolation and HA extraction. Groups of 25× COCs were cultured in 1 ml M199 culture medium under the above described conditions, at intervals from 0 to 48 h. At the end of the culture period, the COCs were washed four times in 500 µl PBS-PVA with their gentle transferral, and oocytes were mechanically removed by repeated pipetting during the last wash. To determine the HA retained in the expanded cumuli, the expanded cumuli were transferred into an Eppendorf tube in 500 µl of PBS-PVA and enzymatically digested by SHH (2 IU/ml; Sigma-Aldrich) at 39°C overnight. Samples of HA isolated from the COCs were stored at –20°C until measurement. After spectrophotometric analysis, the synthesis of HA was expressed as HA concentration (µg/ml) in the solution obtained.

Statistical analysis. All the experiments were repeated at least three times. Data were analyzed using the General Linear Models (GLM) procedure of SAS software (Statistical Analysis System, Version 9.3, 2012). Significant differences between groups with equal and diverse numbers of repetition were determined using the *t*-test and Sheffé's test, respectively. The level of significance was set at $P < 0.05$.

Experimental design

Testing of enzymes with hyaluronidase activity and spectrophotometry optimization. The aim of this experiment was to evaluate the most suitable combination of HA-digesting enzyme and incubation medium. An assessment of spectrophotometric measurement wavelength for maximal absorbance yield was performed. Two enzymes creating heterodimers and oligomers from HA polymer in different ways (with or without involvement of water molecules) in various media were used: (a) non-hydrolytic hyaluronan lyase SHH with β-elimination activity as an enzyme specifically degrading HA (Vanderhyden 1993), with unsaturated bound creation absorbing light

doi: 10.17221/51/2015-CJAS

in a UV absorption spectrum at 232 nm (Yosizawa et al. 1983); (b) concurrently, bovine testicular hyaluronidase (BTH), hydrolytic enzyme creating saturated heterodimer bounds non-absorbing ultraviolet (UV) light (negative control). The presence of HA-degrading products was proved by HPLC. The optimization of spectrophotometry, focused on wavelength finding with maximal yield absorbance, was simultaneously performed.

Verification of measurement of β-elimination products. The aim of this experiment was to prove the absorbance of standards of HA oligomers (HA4–HA10) without enzyme digestion. Concurrently, HA polymer with and without SHH (SHH^+ and SHH^-, respectively) digestion was measured for spectrophotometry specificity to β-elimination products. The absorbance was spectrophotometrically measured at 216 nm.

Verification of HA content according to COC abundance. The aim of this experiment was to verify HA content in dependence on COC number in the 15×, 25× or 50× COCs per sample, prepared after 48 h of *in vitro* cultivation, and to substantiate the specificity of HA analysis to COC abundance-derived production of HA. The HA was spectrophotometrically analyzed at the above optimized wavelength. Simultaneously, an evaluation of oocyte maturation was performed.

Comparison of the cumulus expansion evaluation methods. The aim of the last experiment was to compare the results of visual and analytical methods of cumulus expansion evaluation. Three methods for cumulus expansion assessment were tested on the same population of 25× COCs: visual evaluation of expanded COCs (Vanderhyden 1993), area measurement of COCs (Daen et al. 1994) and, finally, spectrophotometrical HA analysis. For this experiment, the COCs were tested on an 8-h time scale for 48 h. Results from oocyte maturation were simultaneously collected.

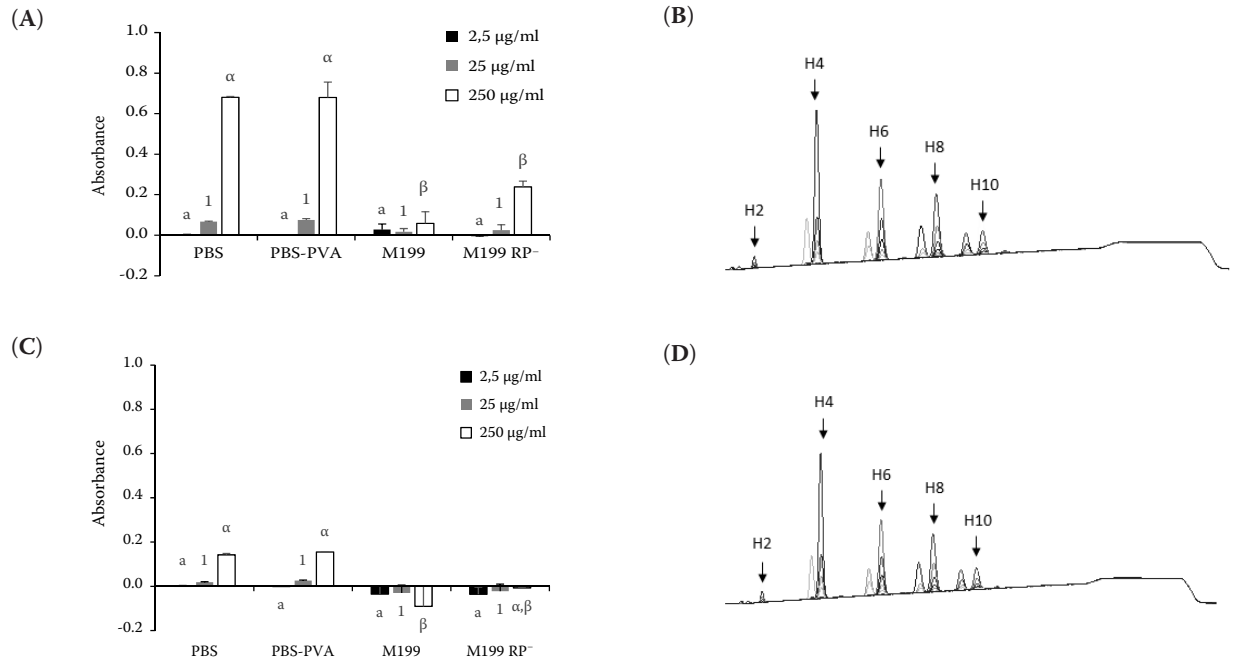


Figure 1. Presence of digested hyaluronic acid (HA) products by hyaluronan lyase from *Streptomyces hyalurolyticus* (SHH) (**A**, **B**) and bovine testicular hyaluronidase (BTH) (**C**, **D**). Comparison of different enzyme–medium combinations for HA digestion and spectrophotometric measurement (wavelength 232 nm). SHH with β-elimination activity (**A**) and hydrolytically digested BTH (**C**) were used. Data were verified using HPLC (210 and 235 nm wavelength for detecting unsaturated and saturated bounds, respectively), all solutions were diluted in PBS and representative chromatograms are shown (**B**, **D**)

bars show the means of three independent experiments ± SEM

GLM procedure followed by *t*-test was performed and different letters indicate significant differences ($P < 0.05$)

PBS = phosphate buffered solution, PBS-PVA = 0.01 % polyvinyl alcohol in phosphate buffered solution, M199 = M199 culture medium, M199 RP^- = M199 without red phenol

doi: 10.17221/51/2015-CJAS

RESULTS

Testing of enzymes with hyaluronidase activity in different media and spectrophotometry optimization. The aim of this experiment was to assess the optimal medium for hyaluronan lyase (SHH) digestion and optimal wavelength for spectrophotometric analysis with maximum absorbance. Based on our results, SHH in PBS has been defined as an optimal combination for real sample preparation. Moreover, PVA addition, necessary for COC manipulation, offers comparable results to SHH-PBS usage. The M199 and M199 without red phenol (M199 RP$^-$) culture media manifested unsuitable conditions for SHH digestion and spectrophotometry analysis, where absorbance values were significantly lower (Figure 1A). At the same time, BTH usage, with presumed no detection of β-elimination products in UV light, was verified and no detectable absorbance was measured (Figure 1C). The detection of HA dimers and HA oligomers, regardless of β-elimination enzyme ability, was revealed by retention time by HPLC (Figure 1B, D). A wavelength of 232 nm in the ultraviolet spectrum was used. Thereafter, the SHH-PBS was selected for standard preparation and a 190–280 nm wavelength was used for optimal wavelength evaluation. The highest absorbance was measured at 216 nm in all the HA standards used (Figure 2), and this wavelength was used in subsequent experiments. The detection limit of spectrophotometry was checked concurrently, and absorbance equalling ~0.13 (i.e. 60 µg/ml HA polymer) was established as the minimal detectable concentration of β-elimination products for spectrophotometry (Figure 2).

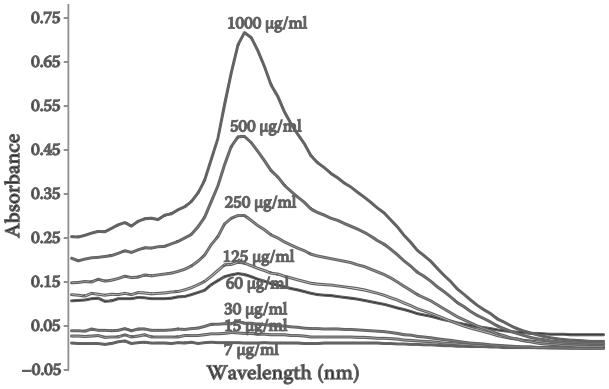


Figure 2. Wavelength optimization of spectrophotometric measurement. The 7–1000 µg/ml HA standard solutions were digested by SHH in PBS and β-elimination products were measured in wavelength range 190–270 nm

HA = hyaluronic acid, SHH = hyaluronan lyase from *Streptomyces hyalurolyticus*, PBS = phosphate buffered solution

Measurement of standard solutions of HA polymer and its oligomers. The standards of HA oligomers (4HA–10HA containing 2–5 glucuronic acid-glycosaminoglycan heterodimers, respectively) were used for verification of the spectrophotometry analysis of digested HA polymer. The present data support previous SHH-digested HA polymer measurements as the result of the presence of HA-digesting products of β-elimination with a comparable absorbance response (Figure 3). Absorbance of non-digested HA polymer was

Table 1. Meiotic maturation of oocytes during *in vitro* cultivation of cumulus-oocyte complexes

	0 h	8 h	16 h	24 h	32 h	40 h	48 h
GV	94.0 ± 8.5^a	96.8 ± 3.9^a	58.7 ± 11.6^b	3.0 ± 2.2^c	1.3 ± 1.3^c	–^{c}	–^{c}
LD	–^{b}	3.2 ± 0.9^b	22.0 ± 6.1^a	1.0 ± 0.5^b	–^{b}	–^{b}	–^{b}
MI	–^{b}	–^{b}	15.7 ± 5^b	91.0 ± 3.8^a	42.7 ± 20.5^{a,b}	29.5 ± 15.3^b	4.0 ± 1.4^b
AI/TI	–^{c}	–^{c}	–^{c}	5.0 ± 5.0^b	19.3 ± 5.0^a	14.9 ± 7.5^a	4.5 ± 2.3^b
MII	–^{c}	–^{c}	–^{c}	–^{c}	36.7 ± 20.8^b	49.2 ± 19.9^b	90.5 ± 4.2^a
Degenerated	6.0 ± 2.6^a	–^{a}	3.6 ± 3.3^a	–^{a}	–^{a}	6.4 ± 5.3^a	1.0 ± 0.4^a
n	75	200	200	250	150	250	250

GV = germinal vesicle oocytes, LD = late diakinesis oocytes, MI = metaphase I oocytes, AI/TI = anaphase I or telophase I oocytes, MII = metaphase II oocytes

data are expressed as a percentage proportion of meiotic stages and show the means of at least three independent experiments ± SEM

GLM procedure followed by Sheffé's test was performed and different letters indicate significant differences in the same stage of meiotic maturation, i.e. in rows ($P < 0.05$)

doi: 10.17221/51/2015-CJAS

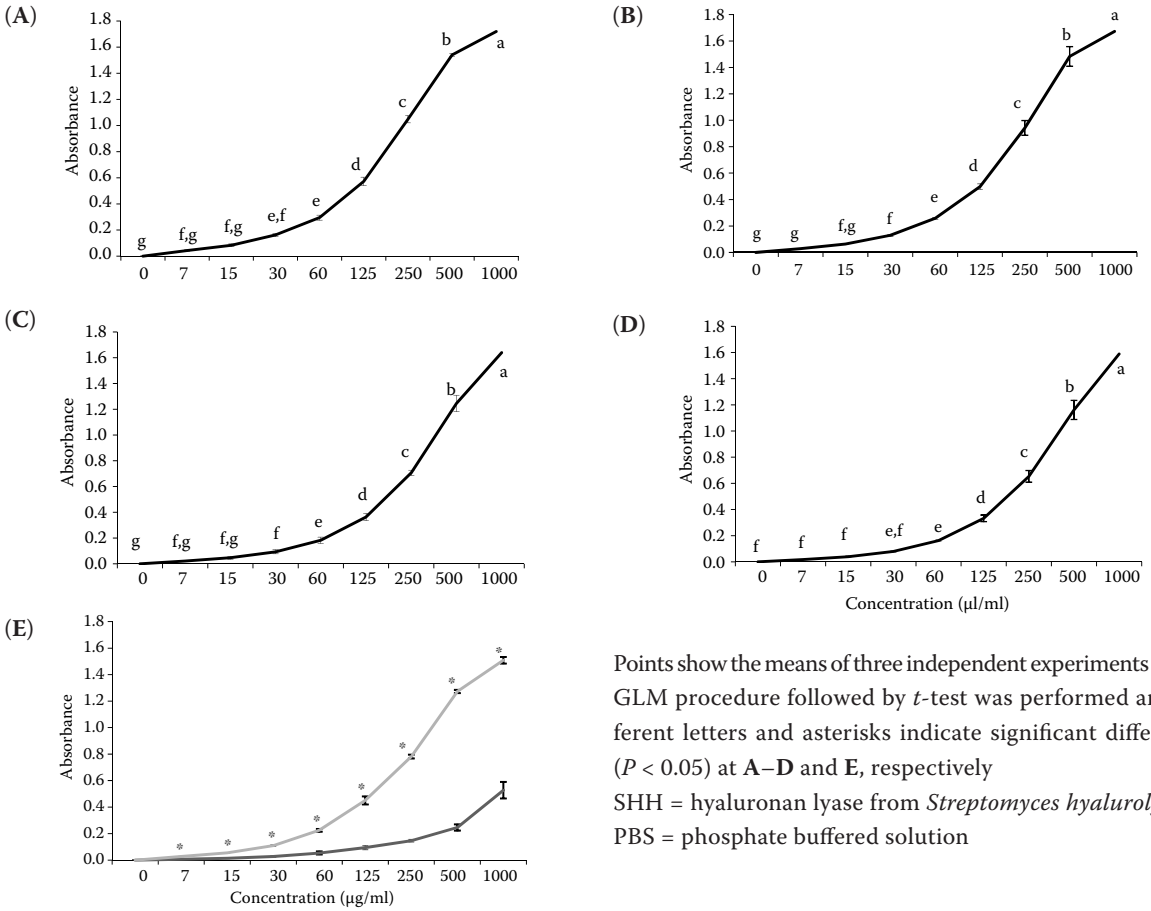


Figure 3. Measurement of standards of undigested hyaluronic acid (HA) oligomers – 4HA (**A**), 6HA (**B**), 8HA (**C**), and 10HA (**D**). The basal undigested HA polymer absorbance was measured (SHH^-) and compared with SHH^+ digestion (**E**). Standard solutions were diluted in PBS and spectrophotometrically measured at 216 nm wavelength

also revealed and weak absorbance was detected. Therefore, approximately 500 µg/ml undigested HA revealed an absorbance value equal to 60 µg/ml of SHH-digested HA polymer (Figure 3E). The standards of 4HA–10HA showed approximately twice as high a detection limit compared to β-elimination products of HA polymer with equal absorbance values (Figure 3A–D).

COC abundance-derived HA content in a sample. The aim of this experiment was to show that HA content is an applicable marker for evaluating cumulus expansion. Therefore, populations of 15×, 25× or 50× COCs were washed and digested for measuring HA retained in COCs after a 48-h *in vitro* cultivation. No significant difference was revealed in HA between the 15× and 25× COCs samples and a significant increase was found in the 50× COCs sample, where HA content showed an almost four-times higher HA sample concentration (213.58 ± 82.15 vs 854.83 ± 113.47 µg/ml for 25× and 50× COCs sample, respectively). The data are summarized in Figure 4.

Comparison of expanded cumulus evaluation during COC in vitro cultivation. Three different methods of expanded cumulus evaluation were tested: (1) classification of expanded cumuli, (2) area measurement of COCs, and (3) HA analysis. All the methods used showed an increasing trend during *in vitro* cultivation of COCs (Figures 5–7). Cumulus classification and area measurement showed 16 h as the first time point with a significant increase of visible expansion and area enlargement, respectively (Figures 5 and 6). On the other hand, no differences were detected in HA content and no significant increase was measured between 16 and 24 h (Figure 7). A further significant increase in HA production was observed at 40–48 h, while cumulus classification and area measurement differences were not seen. At the same time, evaluation of oocyte maturation

doi: 10.17221/51/2015-CJAS

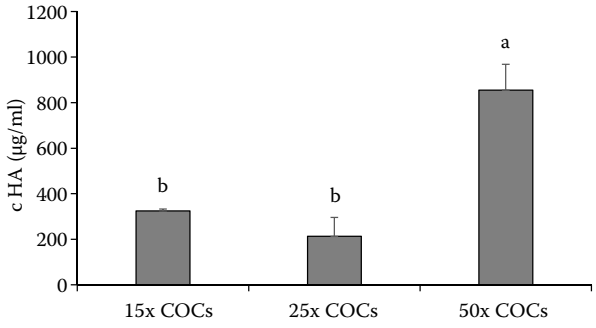


Figure 4. Verification of hyaluronic acid (HA) content depending on cumulus-oocyte complexes (COCs) abundance after a 48-h *in vitro* cultivation. Samples from COCs were prepared using SHH digestion in PBS-PVA overnight. Absorbance measured at 216 nm wavelength and HA concentration (µg/ml) in samples was calculated using polynomic curves of HA standards digested under equal conditions

bars show the means of three independent experiments ± SEM
GLM procedure followed by *t*-test was performed and different letters indicate significant differences ($P < 0.05$)
SHH = hyaluronan lyase from *Streptomyces hyalurolyticus*, PBS-PVA = 0.01 % polyvinyl alcohol in phosphate buffered solution

was verified and the standard course of meiotic maturation was observed (Table 1).

DISCUSSION

An adequate cumulus expansion is essential for successful oocyte maturation. As such, the intensity of cumuli expansion is a biomarker for oocyte quality prediction, key for subsequent *in vitro* fertilization efficiency. Some subjective non-invasive approaches have been used for this purpose (Vanderhyden et al. 1990; Daen et al. 1994). However, some disadvantages (e.g. subjectivity in evaluation, three-dimensional structure non-recognition, inability to indicate cumulus quality) make these methods less attractive. Therefore, a more precise method for the exact study of cumulus expansion and for oocyte quality estimation would be suitable. Analytical measurement of HA, the most abundant compound of expanded cumulus, offers a tool for the objective quantification of cumulus expansion.

In accordance with previous studies (Salustri et al. 1989, 1995; Nakayama et al. 1996), our results indicate that HA, as an abundant compound of

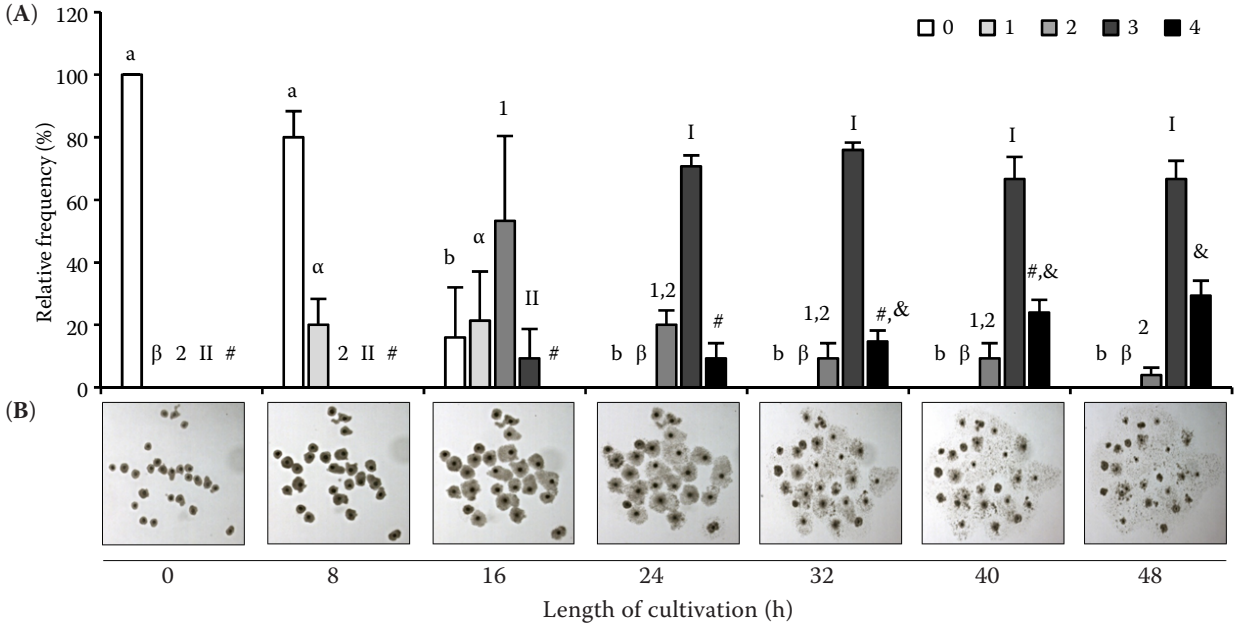


Figure 5. Visual evaluation of expanded cumulus-oocyte complexes (COCs) on an 8-h time scale for 48 h (score 0 = no observable expansion, +1 = minimal observable expansion, +2 = extended expansion to several layers, +3 = complete expansion excluding *corona radiata*, and +4 = complete expansion including *corona radiate*) (**A**); representative images of COCs at each time point (**B**)

data were expressed as a percentage proportion of COC population, bars show the means of three independent experiments ± SEM
GLM procedure followed by *t*-test was performed and different letters indicate significant differences in the same score grade of cumulus expansion ($P < 0.05$)

doi: 10.17221/51/2015-CJAS

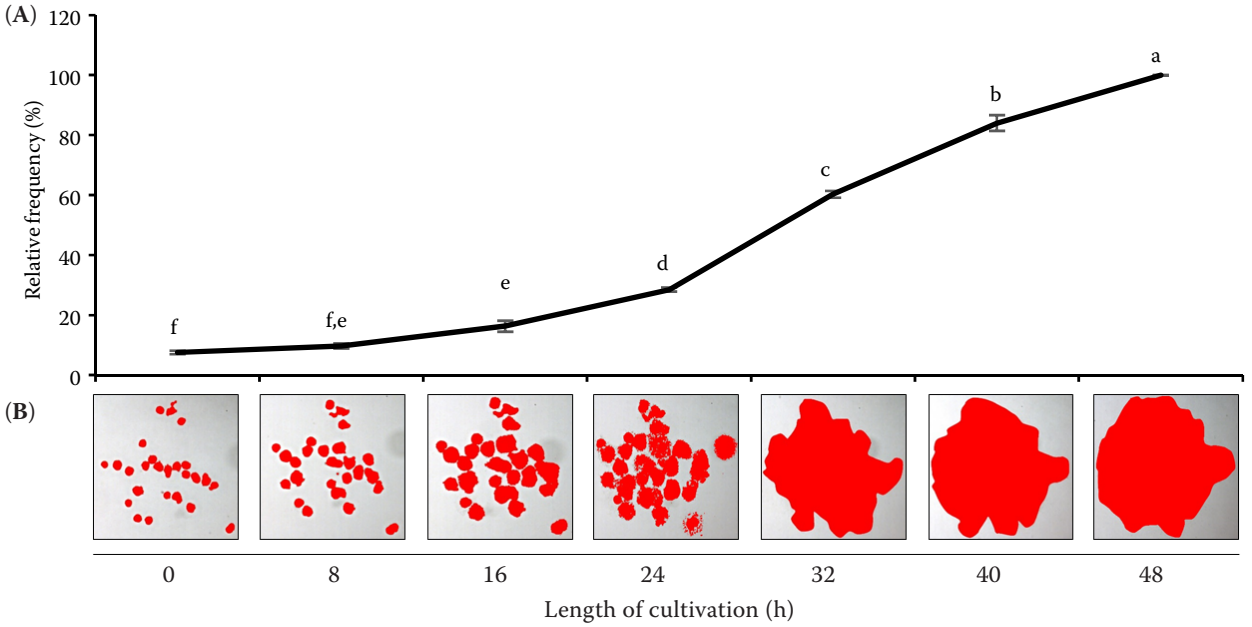


Figure 6. Area measurement of cumulus-oocyte complexes (COCs) on an 8-h time scale for 48 h (**A**); representative images of threshold COCs at each time point (**B**). Data were related to COCs after a 48-h cultivation (i.e. 100%)

points show the means of three independent experiments ± SEM

GLM procedure followed by *t*-test was performed and different letters indicate significant differences ($P < 0.05$)

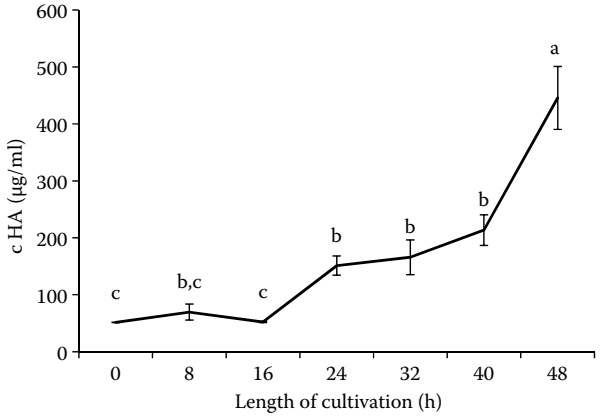


Figure 7. Analysis of hyaluronic acid (HA) retained in cumulus-oocyte complexes (COCs) on an 8-h time scale for 48 h. COCs were prepared using SHH digestion in PBS-PVA overnight. Absorbance was measured at 216 nm wavelength and HA concentration (µg/ml) in samples was calculated using polynomic curves of HA standards digested under equal conditions

points show the means of at least three independent experiments ± SEM

GLM procedure followed by Sheffé's test was performed and different letters indicate significant differences ($P < 0.05$)

SHH = hyaluronan lyase from *Streptomyces hyalurolyticus*, PBS-PVA = 0.01 % polyvinyl alcohol in phosphate buffered solution

expanded cumuli, is thus a possible candidate biomarker of expansion quantity. In our experiments, we used high specific hyaluronan lyase produced by *Streptomyces hyalurolyticus* (Eppig 1980; Vanderhyden 1993), and a mixture of 2HA and longer HA oligomers, in general called β-elimination products, were produced and subsequently measured. Only double bounds of SHH-derived 2HA/HA oligomers are capable of absorbing UV light (Alkrad et al. 2003). However, our spectrophotometric analysis showed lower absorbance values of SHH-digested HA polymer compared with undigested 4-6HA. This observation, supported by HPLC analysis, suggests that SHH digestion of HA polymer loses absorbance in the occurrence of 8–10HA oligomers, and the detection limit of digested HA polymer is slightly decreased. In addition to HPLC improvement, optimization of our measurement wavelength was performed, and 216 nm was established as the optimal wavelength and subsequently used. A different optimal wavelength compared with the initially tested 232 nm has been confirmed by previous studies (Takagaki et al. 1994; Alkrad et al. 2003), which describe various wavelengths for spectrophotometric measurement of HA-digestion product, pointing out divergent laboratory conditions. Overall, the results suggest that the original

doi: 10.17221/51/2015-CJAS

product measured by spectrophotometry after HA digestion equals β-elimination products, and that the real sample from COCs reflects their HA content.

The presented evaluation of cumulus expansion based on HA analysis offers an expression of HA production retained in COCs. We have proven the dependence on COC abundance when HA retaining by COCs significantly increased in samples containing 50× COCs. Although 15× and 25× COCs did not show any differences in the HA content, 25× COCs samples gave a more precise analysis with respect to the variability of COCs. Besides tritium-labelled glycosaminoglycans (Solursh 1976; Eppig 1979), this work supports the measurement of HA production as a usable approach for evaluating cumulus expansion. In spite of radioisotope usage, HA analytical measurement is based on real HA production, without the addition of radioisotope-labelled precursors at the beginning of COC *in vitro* cultivation (Eppig 1979). Moreover, spectrophotometric HA analysis becomes more available due to the absence of radioisotope needs. On the other hand, our suggested method does not respect total HA production and the HA released by COCs into the surrounding medium is not affected, so the method should be further developed.

In our experiments, we compared HA analysis with commonly used cumulus expansion evaluation methods: the subjective classification of COCs and image-derived area measurement, and HA analysis. Based on the results of cumulus expansion classification and area measurement, a different time schedule of cumulus expansion as measured by these two methods should be pointed out. Evidently, area measurement seems to be more consistent through repeated experimentation. On the other hand, this quite consistent and non-invasive simple method of area measurement does not enable the observation of the three-dimensional structure of the expanded cumuli. This phenomenon can be affected by various compounds tested during the *in vitro* COC cultivation. Changes in HA level can suggest modifications of HA binding proteins (HABPs) which are responsible for expanded cumulus structure (Yokoo et al. 2002; Nemcova et al. 2007), resulting in a change in HA quantity and not a change in the visible quality of COCs.

Based on our knowledge, the quality of cumulus expansion can determine oocyte meiotic maturation and its developmental potency (Qian et al. 2003; Feuerstein et al. 2012; Bergandi et al. 2014). Conversely, cumulus expansion physiologically reflects the meiotic stage of oocyte secreting CEEFs (Prochazka et al. 1998; Dragovic et al. 2005). Our findings are in accordance with those when we detected meiosis re-initiation taking place after approximately 16 h of *in vitro* culture, when cumulus expansion was beginning. This observation is reported in previous studies describing the significance of cumulus expansion for re-initiation of oocyte meiosis (Dekel et al. 1981; Chen et al. 1990), as well as the effect of CEEFs on cumulus expansion intensity (Nakayama et al. 1996). In addition to the above-mentioned, further meiotic progress can be regulated by other methods, such as the HA-activated CD44 receptor, one of HABPs (Kimura et al. 2002; Yokoo et al. 2007). As such, following HA production can be required in further meiotic maturation, although cumulus expansion for 24 hours and longer seems to be a passive process as a result of the momentum of primary gonadotropin- or growth factor-initiated cumulus expansion, as previous studies have described (Abeydeera et al. 1998).

This study monitored the course of cumulus expansion together with oocyte maturation in *in vitro* conditions. Cumulus expansion was evaluated by three different methods out of which the quantification of HA seems to be the best method for precise cumulus expansion study and oocyte quality estimation. HA-evaluated cumulus expansion was identified as a suitable biomarker which responds to the stage of meiotic progression of oocyte maturation.

CONCLUSION

Cumulus expansion reflects meiotic maturation and its features refer to the quality and developmental potential of the oocyte. HA-assessed cumulus expansion and its measurement in COCs offer a suitable tool for estimating COC and oocyte quality. This work presents a simply applicable method for the isolation and measurement of HA retained in COCs.

Potentially, HPLC analysis seems to be a helpful alternative when longer digested HA products of β-elimination can be detected, regardless of the efficiency of enzyme digestion. Moreover, a higher sensitivity of HPLC can provide an analysis of HA released by COCs on to the culture medium. Further experiments are needed for verification of these modifications, which could lead to an improvement in COC-produced HA measurement.

doi: 10.17221/51/2015-CJAS

REFERENCES

Abeydeera L.R., Wang W.H., Cantley T.C., Prather R.S., Day B.N. (1998): Presence of beta-mercaptoethanol can increase the glutathione content of pig oocytes matured in vitro and the rate of blastocyst development after in vitro fertilization. Theriogenology, 50, 747–756.

Alkrad J.A., Mrestani Y., Stroehl D., Wartewig S., Neubert R. (2003): Characterization of enzymatically digested hyaluronic acid using NMR, Raman, IR, and UV-Vis spectroscopies. Journal of Pharmaceutical and Biomedical Analysis, 31, 545–550.

Appeltant R., Somfai T., Nakai M., Bodo S., Maes D., Kikuchi K., Van Soom A. (2015): Interactions between oocytes and cumulus cells during in vitro maturation of porcine cumulus-oocyte complexes in a chemically defined medium: effect of denuded oocytes on cumulus expansion and oocyte maturation. Theriogenology, 83, 567–576.

Assidi M., Dieleman S.J., Sirard M.A. (2010): Cumulus cell gene expression following the LH surge in bovine preovulatory follicles: potential early markers of oocyte competence. Reproduction, 140, 835–852.

Assou S., Haouzi D., De Vos J., Hamamah S. (2010): Human cumulus cells as biomarkers for embryo and pregnancy outcomes. Molecular Human Reproduction, 16, 531–538.

Bergandi L., Basso G., Evangelista F., Canosa S., Dalmasso P., Aldieri E., Revelli A., Benedetto C., Ghigo D. (2014): Inducible nitric oxide synthase and heme oxygenase 1 are expressed in human cumulus cells and may be used as biomarkers of oocyte competence. Reproductive Sciences, 21, 1370–1377.

Chen L., Wert S.E., Hendrix E.M., Russell P.T., Cannon M., Larsen W.J. (1990): Hyaluronic acid synthesis and gap junction endocytosis are necessary for normal expansion of the cumulus mass. Molecular Reproduction and Development, 26, 236–247.

Daen F.P., Sato E., Naito K., Toyoda Y. (1994): The effect of pig follicular fluid fractions on cumulus expansion and male pronucleus formation in porcine oocytes matured and fertilized in vitro. Journal of Reproduction and Fertility, 101, 667–673.

Davachi N.D., Kohram H., Zainoaldini S. (2012): Cumulus cell layers as a critical factor in meiotic competence and cumulus expansion of ovine oocytes. Small Ruminant Research, 102, 37–42.

Dekel N., Hillensjo T., Kraicer P.F. (1979): Maturational effects of gonadotropins on the cumulus-oocyte complex of the rat. Biology of Reproduction, 20, 191–197.

Dekel N., Lawrence T.S., Gilula N.B., Beers W.H. (1981): Modulation of cell-to-cell communication in the cumulus-oocyte complex and the regulation of oocyte maturation by LH. Developmental Biology, 86, 356–362.

Dragovic R.A., Ritter L.J., Schulz S.J., Amato F., Armstrong D.T., Gilchrist R.B. (2005): Role of oocyte-secreted growth differentiation factor 9 in the regulation of mouse cumulus expansion. Endocrinology, 146, 2798–2806.

Eppig J.J. (1979): FSH stimulates hyaluronic acid synthesis by oocyte-cumulus cell complexes from mouse preovulatory follicles. Nature, 281, 483–484.

Eppig J.J. (1980): Role of serum in FSH stimulated cumulus expansion by mouse oocyte-cumulus cell complexes in vitro. Biology of Reproduction, 22, 629–633.

Fagbohun C.F., Downs S.M. (1990): Maturation of the mouse oocyte-cumulus cell complex: stimulation by lectins. Biology of Reproduction, 42, 413–423.

Feuerstein P., Puard V., Chevalier C., Teusan R., Cadoret V., Guerif F., Houlgatte R., Royere D. (2012): Genomic assessment of human cumulus cell marker genes as predictors of oocyte developmental competence: impact of various experimental factors. PLoS One, 7, e40449.

Flechon J.E., Degrouard J., Kopecny V., Pivko J., Pavlok A., Motlik J. (2003): The extracellular matrix of porcine mature oocytes: origin, composition and presumptive roles. Reproductive Biology and Endocrinology, 1, 124.

Huang Z., Wells D. (2010): The human oocyte and cumulus cells relationship: new insights from the cumulus cell transcriptome. Molecular Human Reproduction, 16, 715–725.

Kimura N., Konno Y., Miyoshi K., Matsumoto H., Sato E. (2002): Expression of hyaluronan synthases and CD44 messenger RNAs in porcine cumulus-oocyte complexes during in vitro maturation. Biology of Reproduction, 66, 707–717.

Kubo N., Cayo-Colca I.S., Miyano T. (2015): Effect of estradiol-17β during in vitro growth culture on the growth, maturation, cumulus expansion and development of porcine oocytes from early antral follicles. Animal Science Journal, 86, 251–259.

Machado M.F., Caixeta E.S., Sudiman J., Gilchrist R.B., Thompson J.G., Lima P.F., Price C.A., Buratini J. (2015): Fibroblast growth factor 17 and bone morphogenetic protein 15 enhance cumulus expansion and improve quality of in vitro-produced embryos in cattle. Theriogenology, 84, 390–398.

McKenzie L.J., Pangas S.A., Carson S.A., Kovanci E., Cisneros P., Buster J.E., Amato P., Matzuk M.M. (2004): Human cumulus granulosa cell gene expression: a predictor of fertilization and embryo selection in women undergoing IVF. Human Reproduction, 19, 2869–2874.

Motlik J., Fulka J. (1976): Breakdown of the germinal vesicle in pig oocytes in vivo and in vitro. Journal of Experimental Zoology, 198, 155–162.

doi: 10.17221/51/2015-CJAS

Nakayama T., Inoue M., Sato E. (1996): Effect of oocytectomy on glycosaminoglycan composition during cumulus expansion of porcine cumulus-oocyte complexes cultured in vitro. Biology of Reproduction, 55, 1299–1304.

Nemcova L., Nagyova E., Petlach M., Tomanek M., Prochazka R. (2007): Molecular mechanisms of insulin-like growth factor 1 promoted synthesis and retention of hyaluronic acid in porcine oocyte-cumulus complexes. Biology of Reproduction, 76, 1016–1024.

Nevoral J., Orsak M., Klein P., Petr J., Dvorakova M., Weingartova I., Vyskocilova A., Zamostna K., Krejcova T., Jilek F. (2014): Cumulus cell expansion, its role in oocyte biology and perspectives of measurement: A review. Scientia Agriculturae Bohemica, 45, 212–225.

Prochazka R., Nagyova E., Brem G., Schellander K., Motlik J. (1998): Secretion of cumulus expansion-enabling factor (CEEF) in porcine follicles. Molecular Reproduction and Development, 49, 141–149.

Prochazka R., Petlach M., Nagyova E., Nemcova L. (2011): Effect of epidermal growth factor-like peptides on pig cumulus cell expansion, oocyte maturation, and acquisition of developmental competence in vitro: comparison with gonadotropins. Reproduction, 141, 425–435.

Qian Y., Shi W.Q., Ding J.T., Sha J.H., Fan B.Q. (2003): Predictive value of the area of expanded cumulus mass on development of porcine oocytes matured and fertilized in vitro. Journal of Reproduction and Development, 49, 167–174.

Salustri A., Yanagishita M., Hascall V.C. (1989): Synthesis and accumulation of hyaluronic acid and proteoglycans in the mouse cumulus cell-oocyte complex during follicle-stimulating hormone-induced mucification. Journal of Biological Chemistry, 264, 13840–13847.

Salustri A., Camaioni A., Tirone E., D'Alessandris C. (1995): Hyaluronic acid and proteoglycan accumulation in the cumulus oophorus matrix. Italian Journal of Anatomy and Embryology, 100, 479–484.

Solursh M. (1976): Glycosaminoglycan synthesis in the chick gastrula. Developmental Biology, 50, 525–530.

Takagaki K., Takeda Y., Nakamura T., Daidouji K., Narita H., Endo M. (1994): Analysis of glycosaminoglycans by high-performance liquid chromatography. Journal of Biochemical and Biophysical Methods, 28, 313–320.

Vanderhyden B.C. (1993): Species differences in the regulation of cumulus expansion by an oocyte-secreted factor(s). Journal of Reproduction and Fertility, 98, 219–227.

Vanderhyden B.C., Caron P.J., Buccione R., Eppig J.J. (1990): Developmental pattern of the secretion of cumulus expansion-enabling factor by mouse oocytes and the role of oocytes in promoting granulosa cell differentiation. Developmental Biology, 140, 307–317.

Volpi N. (2000): Hyaluronic acid and chondroitin sulfate unsaturated disaccharides analysis by high-performance liquid chromatography and fluorimetric detection with dansylhydrazine. Analytical Biochemistry, 277, 19–24.

Yokoo M., Tienthai P., Kimura N., Niwa K., Sato E., Rodrigues-Martinez H. (2002): Localisation of the hyaluronan receptor CD44 in porcine cumulus cells during in vivo and in vitro maturation. Zygote, 10, 317–326.

Yokoo M., Shimizu T., Kimura N., Tunjung W.A., Matsumoto H., Abe H., Sasada H., Rodriguez-Martinez H., Sato E. (2007): Role of the hyaluronan receptor CD44 during porcine oocyte maturation. Journal of Reproduction and Development, 53, 263–270.

Yokoo M., Kimura N., Sato E. (2010): Induction of oocyte maturation by hyaluronan-CD44 interaction in pigs. Journal of Reproduction and Development, 56, 15–19.

Yosizawa Z., Ototani N., Satake S. (1983): A simple method for the quantitation of glycuronic acid-containing glycosaminoglycans with mucopolysaccharidases. Analytical Biochemistry, 128, 250–256.

Yuan Y., Ida J.M., Paczkowski M., Krisher R.L. (2011): Identification of developmental competence-related genes in mature porcine oocytes. Molecular Reproduction and Development, 78, 565–575.

Zhang M., Tao Y., Xia G., Xie H., Hong H., Wang F., Lei L. (2005): Atrial natriuretic peptide negatively regulates follicle-stimulating hormone-induced porcine oocyte maturation and cumulus expansion via cGMP-dependent protein kinase pathway. Theriogenology, 64, 902–916.

Received: 2015–06–30
Accepted after corrections: 2015–12–14

Corresponding Author

Ing. Kateřina Zámostná, Czech University of Life Sciences Prague, Faculty of Agrobiology, Food and Natural Resources, Department of Veterinary Sciences, Kamýcká 129, 165 21 Prague 6-Suchdol, Czech Republic
Phone: +420 224 382 932, e-mail: zamostna@af.czu.cz

Nitric Oxide 51 (2015) 24–35

Contents lists available at ScienceDirect

Nitric Oxide

journal homepage: www.elsevier.com/locate/yniox

Endogenously produced hydrogen sulfide is involved in porcine oocyte maturation *in vitro*

Jan Nevoral [a,*], Tereza Žalmanová [a], Kateřina Zámostná [a], Tomáš Kott [b], Veronika Kučerová-Chrpová [a], Jean-Francois Bodart [c], Armance Gelaude [c], Radek Procházka [d], Matyáš Orsák [e], Miloslav Šulc [e], Pavel Klein [f], Markéta Dvořáková [a], Ivona Weingartová [a], Aurélia Víghová [a], Kristýna Hošková [a], Tereza Krejčová [a], František Jílek [a], Jaroslav Petr [b]

[a] Czech University of Life Sciences in Prague, Faculty of Agrobiology, Food and Natural Resources, Department of Veterinary Sciences, Prague, Czech Republic
[b] Research Institute of Animal Production, Prague, Czech Republic
[c] Université Lille1, Sciences et Technologies, Team Régulation des Signaux de Division, UMR 8576 CNRS, Villeneuve d'Ascq Cedex, France
[d] Institute of Animal Physiology and Genetics, Czech Academy of Sciences, Liběchov, Czech Republic
[e] Czech University of Life Sciences in Prague, Faculty of Agrobiology, Food and Natural Resources, Department of Chemistry, Prague, Czech Republic
[f] Charles University in Prague, Faculty of Medicine in Pilsen, Biomedical Centre, Pilsen, Czech Republic

ARTICLE INFO

Article history:
Received 24 July 2015
Received in revised form
12 September 2015
Accepted 29 September 2015
Available online 9 October 2015

Keywords:
Oocyte
GVBD
Meiotic maturation
Cumulus expansion
Gasotransmitter
Hydrogen sulfide

ABSTRACT

Hydrogen sulfide, one of three known gasotransmitters, is involved in physiological processes, including reproductive functions. Oocyte maturation and surrounding cumulus cell expansion play an essential role in female reproduction and subsequent embryonic development. Although the positive effects of exogenous hydrogen sulfide on maturing oocytes are well known, the role of endogenous hydrogen sulfide, which is physiologically released by enzymes, has not yet been described in oocytes. In this study, we observed the presence of Cystathionine β-Synthase (CBS), Cystathionine γ-Lyase (CTH) and 3-Mercaptopyruvate Sulfurtransferase (3-MPST), hydrogen sulfide-releasing enzymes, in porcine oocytes. Endogenous hydrogen sulfide production was detected in immature and matured oocytes as well as its requirement for meiotic maturation. Individual hydrogen sulfide-releasing enzymes seem to be capable of substituting for each other in hydrogen sulfide production. However, meiosis suppression by inhibition of all hydrogen sulfide-releasing enzymes is not irreversible and this effect is a result of M-Phase/Maturation Promoting Factor (MPF) and Mitogen-Activated Protein Kinase (MAPK) activity inhibition. Futhermore, cumulus expansion expressed by hyaluronic acid (HA) production is affected by the inhibition of hydrogen sulfide production. Moreover, quality changes of the expanded cumuli are indicated. These results demonstrate hydrogen sulfide involvement in oocyte maturation as well as cumulus expansion. As such, hydrogen sulfide appears to be an important cell messenger during mammalian oocyte meiosis and adequate cumulus expansion.

1. Introduction

Meiosis in mammalian oocytes is spontaneously arrested at the dictyotene of prophase I. Prior to this, growing oocytes synthesise large amounts of protein essential for the resumption of this meiotic block, which is followed by oocyte meiosis [1]. A fully-grown dictyate oocyte, often called a germinal vesicle (GV) oocyte, holds the meiotic block until gonadotropin stimulation *in vivo* and *in vitro* conditions, when the re-initiation of oocyte meiosis is manifested as germinal vesicle breakdown (GVBD). GVBD is followed by further stages of meiosis I and the establishment of the second meiotic block at metaphase II (MII). The process that begins with GVBD and continues through meiosis I to MII is called meiotic maturation. Oocyte maturation is necessary for fertilisation ability and successful embryonic development [2]. Therefore, oocyte maturation is a key factor in female fertility as well as in

* Corresponding author. Czech University of Life Sciences in Prague, Kamycka 129, 16521 Prague 6-Suchdol, Czech Republic.
E-mail address: nevoral@af.czu.cz (J. Nevoral).

http://dx.doi.org/10.1016/j.niox.2015.09.007
1089-8603/

assisted reproductive therapy.

Cumulus cells surround the oocyte, and together, they create a cumulus–acolyte complex (COC). Oocyte maturation is accompanied by an increased production of extracellular matrix compounds in the cumulus cells mass [3,4]. COC enlargement associated with mucification, known as cumulus expansion, is based on the production of glycosaminoglycans, especially hyaluronic acid [5,6]. Expanded cumuli prevents the flow of certain meiosis inhibiting factors to the oocyte [7,8]. Moreover, HA itself acts as a signal molecule regulating oocyte maturation [9,10]. These changes result in the activation of key cell cycle kinases that are pivotal for oocyte maturation: M-Phase/Maturation Promoting Factor (MPF) and Mitogen-Activated Protein Kinase (MAPK) [11,12]. The ways in which up-stream factors regulate MPF/MAPK-driven oocyte maturation are not yet fully understood. Based on our recent observations, a potent group of gasotransmitters, gaseous molecules with second messenger action, are evidently involved in the regulation of oocyte metabolism [13,14].

Of the three described gasotransmitters – nitric oxide, hydrogen sulfide and carbon monoxide, only the role of nitric oxide has been more detail studied in oocyte maturation and cumulus expansion [summarised in Ref. [15]]. In addition to nitric oxide, endogenously released hydrogen sulfide is also required for the regulation of many cell functions [summarised in Refs. [16,17]]. However, the role of hydrogen sulfide in oocyte maturation and cumulus expansion remains unclear. Nevertheless, the accelerational effect of sodium sulfide, an exogenous hydrogen sulfide donor, on MPF/MAPK activity and oocyte maturation was recently described. Moreover, this hydrogen sulfide donor also influences hyaluronic acid (HA) production and cumulus expansion [13]. This indicates that endogenous production of hydrogen sulfide may play a physiological role in the regulation of oocyte maturation and cumulus expansion.

Endogenously releasing hydrogen sulfide from amino acid L-cysteine is catalysed by pyridoxal phosphate-dependent enzymes: Cystathionine β-Synthase (CBS), Cystathionine γ-Lyase (CTH) and/or 3-Mercaptopyruvate Sulfurtransferase (3-MPST) [18,19]. CBS is the only hydrogen sulfide-releasing enzyme to be observed in ovarian follicles [20,21]. Neither the physiological production of hydrogen sulfide nor the presence of CTH and 3-MPST has been indicated in oocytes.

We hypothesise that three hydrogen sulfide-releasing enzymes, CBS, CTH and 3-MPST, are present in mammalian oocytes and produce endogenous hydrogen sulfide, which is involved in the regulation of oocyte maturation and cumulus expansion. The aims of the present study consisted of the following: 1) to detect mRNA for CBS, CTH and 3-MPST in pig oocytes and cumulus cells; 2) to localise CBS, CTH and 3-MPST proteins in pig oocytes during oocyte maturation; 3) to demonstrate the effects of inhibitors of these hydrogen sulfide-releasing enzymes on pig oocyte maturation; 4) to measure MPF and MAPK activity in oocytes; and 5) to evaluate cumulus expansion based on HA production.

2. Materials and methods

2.1. Oocyte isolation and in vitro maturation

Porcine ovaries were obtained from 6- to 8-month-old non-cycling gilts (a crossbreed of Landrace x Large White), at the local slaughterhouse (Jatky Plzen a.s., Plzen, Czech Republic) and kept at 39 °C until arrival to the laboratory. 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 selected for 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 International B.V., Boxmeer, Holland) and 5% (v/v) foetal bovine serum (Sigma–Aldrich, USA). The culture medium contained oxamic acid, DL-propargylglycine and/or α-ketoglutaric acid, specific inhibitors of CBS, CTH and 3-MPST, respectively, in double- or triple-combination. An effective concentration of the triple-combination of inhibitors (2 mM oxamic acid, 2 mM DL-propargylglycine and 5 mM α-ketoglutaric acid dissolved in M199 medium; $3C_i$) was used for subsequent experiments. The COCs were matured for 16–48 h in 4-well Petri dishes (Nunc, Fisher Scientific, USA) containing 1.0 ml of culture medium, at 39 °C in a mixture of 5.0% CO_2 in air.

2.2. Assessment of oocyte meiotic maturation

At the end of culture, the COCs were treated with 0.1% 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. Subsequently, the oocytes were 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 and Fulka [22]: GV – germinal vesicle, LD – late diakinesis, MI – metaphase I, AITI – anaphase I to telophase I transition, MII – metaphase II.

2.3. Real time RT-qPCR analysis

The samples for quantitative Real Time RT-qPCR analysis of CBS, CTH and 3-MPST mRNAs were prepared from growing oocytes, fully grown immature (GV), maturing (MI) and matured (MII) oocytes. Concurrently, cumulus cells were used for the same analysis.

RNA was isolated using a NucleicAcid PrepStation 6100 (Applied Biosystems, Fisher Scientific, USA) in accordance with the instruction manual. Total mRNA was transcribed to cDNA with a High-Capacity cDNA Achieve kit (Applied Biosystems, USA) in accordance with manufacture instructions. cDNA was synthesised in a final volume of 100 μl. Sets of specific primers were synthesised in accordance with known sequences to amplify specific products for CBS, CTH and 3-MPST (Table 1).

Real-time PCR was performed using a standard Taq-Man PCR kit protocol (Applied Biosystems, USA). Each PCR reaction was performed in triplicate in a total volume of 10 μl with a 500 nM gene-specific primer and 200 nM TaqMan MGB probes, 5 μl of 2× concentrated Fast TaqMan Universal Master Mix (Applied Biosystems, USA), 1 μl cDNA, and nuclease-free water up to volume. The 7500 Fast Real-Time PCR System (Life Technologies, USA) was utilised for RT-qPCR reactions and the programme used was as follows: 95 °C for 20 s followed by 40 cycles of 95 °C for 2 s and 60 °C for 20 s.

The relative quantification of mRNA expression for each enzyme was determined with data from SDS software using the arithmetical formula $2^{-\Delta\Delta CT}$, according to the comparative Ct method [23], representing the amount of target, normalised to the GAPDH endogenous control as reference [24] and related to fully grown GV oocytes and their cumulus cells.

2.4. Immunocytochemistry and image analysis

GV, MI and MII oocytes were denuded from cumulus cells, fixed and processed as early described Yi et al. [25]. Briefly, oocytes were

Table 1
Specific primers for mRNA of hydrogen sulfide enzymes detection.

Gene	Forward primers 5′–3′	Reverse primers 5′–3′	TaqMan probe 5′–3′	Gene bank accession number	Annealing temperature (°C)	Product size (bp)
CBS	CAGCGCTGCGTGGTGAT	TCACTCAGGAACTTGGACATGTAGTT	CTGCCAGACTCTGTG	XM_006724057	60	62
CTH	GAGCAGTGGGCCTCCAAAG	TTGTTTGAACGTGGTGGACAGT	TGTAGTGCCCCCCATC	NM_001044585	60	60
3-MPST	GCCCGCCGAGTTCCA	TGATGTCCTCGTAGGTCTTGACA	CTGTGCTGGACCCC	XM_001926451	60	62

incubated with rabbit polyclonal anti-CBS (ab96252, 1:200; Abcam, UK), rabbit polyclonal anti-CTH (sc-135203, 1:200; Santa-Cruz Biotechnology, USA) or rabbit polyclonal anti-3-MPST (ab85377, 1:200; Abcam, UK) overnight at 4 °C. Subsequently, the oocytes were washed twice before being incubated with fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit (GAR) IgG (1:200; Invitrogen, Fisher Scientific, USA) for 40 min at room temperature. Thereafter, the oocytes were washed twice and mounted into a Vectashield with 4′6'-diamidino-2-phenylindole (DAPI; Vector Laboratories, USA). Images were acquired using the Ti–U microscope (Nikon, Japan) with Clara Interline CCD camera (Andor Technology PLC, Northern Ireland) operand by NIS Elements Ar software (Nikon, Japan). Negative controls were performed by omitting specific antibodies and these slides were processed at comparable settings. Image analysis, along with 2D deconvolution, was performed by NIS Elements and signal intensity of CBS, CTH or 3-MPST, reduced by a basal signal intensity of appropriate negative control processed by equal procedures.

2.5. Western blotting

GV, MI and MII oocytes and their cumulus cells were used for western blot analysis. Samples were prepared and processed using the method set out by Tumova et al. [26], with slight modifications. In brief, after oocyte denudation, oocytes and cumulus cells were separately lysed in 20 μl of Laemmli buffer containing Triton-X-100 (0.003%, v/v) and SDS (0.001%, v/v), enriched with Complete Mini Protease Inhibitor Cocktail (Roche, Switzerland). Samples were boiled and subjected to SDS-PAGE electrophoresis in 12.5% separating gels and blotted onto a nitrocellulose membrane (GE Healthcare Life Sciences, Amersham, UK). After overnight blocking in 2% non-fat milk in PBS with 0.1% Tween-20 (PBS-T), the membrane was incubated with rabbit polyclonal anti-CBS (ab96252, 1:500; Abcam, UK), rabbit polyclonal anti-CTH (sc-135203, 1:500; Santa-Cruz Biotechnology, USA) or rabbit polyclonal anti-3-MPST (ab85377, 1:500; Abcam, UK) diluted in PBS-T for 60 min at room temperature. Subsequently, the membrane was incubated with horseradish peroxidase (HRP)-conjugated goat anti rabbit IgG in PBS-T (1:120,000; Invitrogen, USA) for 60 min at room temperature. CBS, CTH and 3-MPST recombinant proteins (H00000875–P01, H00001491–P01 and H00004357–P01, respectively; Abnova, Taiwan), with defined molecular weight, were used as a positive control for evidence of oocyte/cumulus CBS/CTH/3-MPST. Proteins were detected using the ECL Select Western Blotting Detection Reagent (GE Healthcare Life Sciences, Amersham, UK) and visualised by a C-Digit Blot Scanner (LI-COR Biosciences, USA).

2.6. Histone H1 and myelin basic protein double assay

The COCs were matured for 16–36 h with or without the presence of the $3C_i$. At each alloted time interval during the culture, COCs were denuded and 15 oocytes per sample were collected. Assays were performed according to the protocol of Kubelka et al. [11], with slight modifications. The oocytes were washed four times in PBS–PVA, 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-glycerophosphate, 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 at −80 °C until assays were performed. An assay of MPF and MAPK activity by their capacity to phosphorylate external substrates, histone H1 (H1) and Myelin Basic Protein (MBP), respectively, was performed. The kinase reaction was initiated by the 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-glycerophosphate, 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-32P]ATP (GE Healthcare Life Sciences, Amersham, 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 Coomassie Blue R250, destained overnight, dried and autoradiographed. Phosphorylated histone H1 and MBP signals were visualised by MultiGauge 2.0 software (FUJI FILM, Japan) and related to metaphase I oocytes of the control group after 24 h cultivation, where we expected the peak of kinase activity [13].

2.7. Spectrophotometric analysis of hydrogen sulfide production

Hydrogen sulfide production was evaluated by spectrophotometry in accordance with Abe and Kimura [27] with modifications due to porcine oocytes. Briefly, immature GV oocytes and matured MII oocytes after 48 h cultivation were denuded and divided into control and inhibited groups (150 oocytes per sample), with or without the addition of $3C_i$, respectively. The oocytes were processed to cell lysate by mechanical disruption in 500 μl cold reaction mixture consisting of 20 mM pyridoxal 5-phosphate and 1 mM L-cysteine in deionised water. After oocyte lysate preparation, 250 μl 1% zinc acetate was added and enzymatic hydrogen sulfide production was initiated by increasing the temperature to 39 °C. After 60 min incubation in an N_2 atmosphere, 250 μl 50% trichloroacetic acid was added to ensure the enzymes were deactivated. The reaction mixture was incubated for the next 60 min under the same conditions, thereafter, 133 μl of 20 mM N,N dimethyl-p-phenylenediamine sulphate (in 7.2 M HCl) and 130 μl of 30 mM $FeCl_3$ (in 1.2 M HCl). The reaction mixture was centrifuged at 10,000 rpm for 1 min and spectrophotometrically measured on a 96-well microtitration plate (wavelength 670 nm) to blank, using a Rainbow ELISA plate reader. The quadratic calibration curve was based on five $Na_2S.9H_2O$ standards (35–600 μM) and used for calculating the amount of lysate hydrogen sulfide. The data was analysed using SoftMax 5.1 software (Molecular Devices, USA) and expressed relative to the control oocyte lysate without the use of $3C_i$.

2.8. Hyaluronic acid (HA) measurement and evaluation of cumulus expansion

Groups of 25 COCs were cultured for 24 or 48 h in 1 ml M199

medium with or without the $3C_i$. At the end of the culture period, the COCs were washed four times in 500 μl PBS–PVA by gentle transferral, and the oocytes were mechanically removed by repeated pipetting during the last washing. To determine the HA retained in the expanded cumuli, the last washing was quantitatively transferred into an eppendorf tube and enzymatically digested by lyase from *Streptomyces hyalurolyticus* (2 IU/ml; Sigma–Aldrich, USA) at 39 °C overnight. Samples of retained HA in the COCs were stored at −20 °C until use.

At the same time, samples were prepared to quantify the amount of HA released by the COCs into the surrounding media. To do so, 900 μl of used culture medium was incubated with 5 μl of Alcalase 2.4 L (2.4 IU/g; Sigma–Aldrich, USA) at 39 °C overnight and passed through Ultracel-30 Microcon microfilters (Merck, USA) by centrifugation for 30 min at 14,000 rpm, 4 °C. High molecular weight HA held on the filter membrane was digested by lyase under the above described conditions. Using enzymatic digestion, HA fragments were washed out from the filter membrane using 200 μl PBS by centrifugation for 30 min at 14,000 rpm, 4 °C, thus being four times more concentrated compared with the original HA concentration in M199. Samples were stored at −20 °C until use.

Subsequently, the solutions were spectrophotometrically measured using Helios Gamma (Fisher Scientific, USA) at 216 nm against blank consisting of PBS–PVA or PBS with lyase, for retained

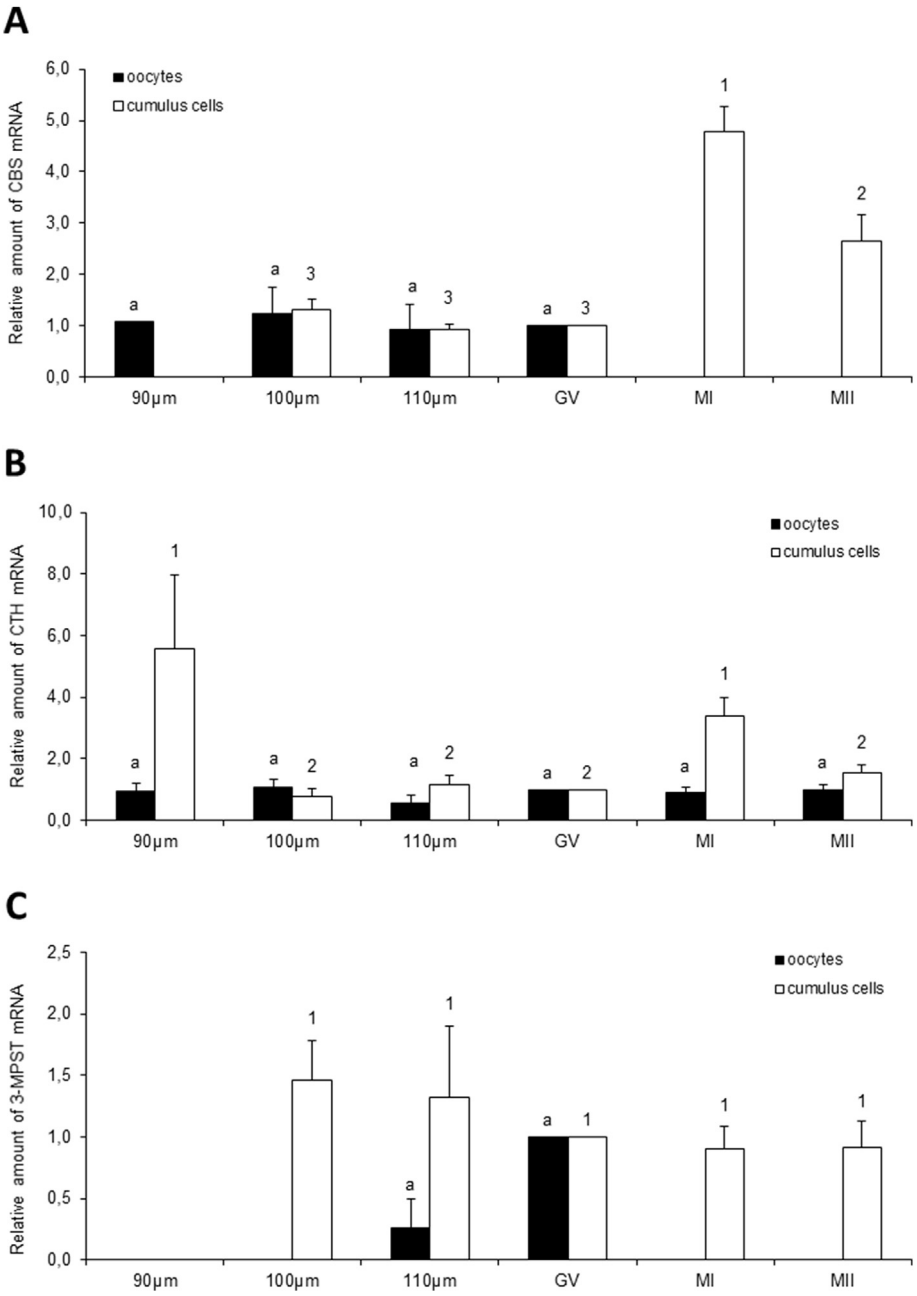


Fig. 1. Amount of mRNA hydrogen sulfide-releasing enzymes in oocytes. A,B,C: The mRNA of CBS, CTH and 3-MPST, respectively, were analysed by real time RT-qPCR in oocytes and their cumulus cells during oocyte growth (with 90 μm, 100 μm and 110 μm diameter), immature GV, maturing MI and matured MII oocytes. The relative mRNA levels were normalised to GAPDH and related to GV oocytes and their cumulus cells by $2^{-\Delta\Delta CT}$ formula. Each stage includes 50 oocytes and their cumulus cells in six independent experiments. The bars show the means ± SEMs of relative mRNA amount. Bars with different letters and numbers are significantly different for oocytes and their cumulus cells, respectively. The GLM procedure followed by Scheffe's test in SAS software was performed for oocyte and cumulus cells separately and groups were tested against each other ($P < 0.05$).

HA and released HA respectively. Disposable polypropylene semi-micro cuvettes (7592 00, PlastiBRAND; Fisher Scientific, USA) were used. The quadratic calibration curve was based on five HA standards (0.006–0.1% sodium hyaluronate, S0780000; Sigma–Aldrich, USA) diluted and digested by protocol used for samples. Concentration of HA was expressed either as the retained or released HA and related to the control group.

2.9. Statistical analysis

The general linear models (GLM) procedure in SAS software (SAS Institute, USA) was used to analyse data from all experiments. Significant differences among groups were determined using Scheffe's test. $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. Expression of hydrogen sulfide-releasing enzymes' genes in porcine oocytes

The aim of the experiment was to demonstrate mRNA presence of hydrogen sulfide-releasing enzymes, CBS, CTH and 3-MPST, using real time RT-qPCR. The mRNA measurement in growing (90, 100 and 110 μm) and immature fully grown oocytes (GV) was performed to demonstrate real CBS, CTH and 3-MPST gene expression as a background for subsequent oocyte maturation (MI, MII) where the mRNA presence was also revealed. mRNA CBS was observed in growing and GV oocytes only, not in maturing (MI) and matured (MII) oocytes. Conversely in cumulus cells, mRNA CBS was found from 110 μm through the period of the oocyte growing and maturing, with a significant increase around meiosis re-initiation (Fig. 1A). A constant level of CTH mRNA was demonstrated in oocytes, at the same time as a fall and rise again in cumulus cells during growth and meiosis re-initiation, respectively (Fig. 1B). Looking at 3-MPST mRNA, its oocyte level was observed only in 110 μm and fully grown GV oocytes, while mRNA was constant in cumulus cells from 100 μm through oocyte growth and maturation (Fig. 1C).

3.2. Observation of hydrogen sulfide-releasing enzymes in porcine oocytes

An objective of this experiment was to demonstrate CBS, CTH and 3-MPST proteins using immunocytochemistry and western blot analysis. For these analyses, immature GV oocytes, *in vitro* maturing MI and matured MII oocytes were used. The presence of CBS, CTH and 3-MPST was demonstrated in all oocyte stages by both immunocytochemistry and western blot (Fig. 2A,B). In addition, the presence of hydrogen sulfide-releasing enzymes was shown in surrounding cumulus cells (Fig. 2B). The presence of CBS and CSE in oocytes seemed to be constant throughout oocyte maturation. In contrast, 3-MPST showed a decrease in protein amount. All hydrogen sulphide-releasing enzymes in cumulus cells exhibited no noticeable change.

3.3. Hydrogen sulfide-releasing enzymes' inhibition suppresses maturation of porcine oocytes throughout MPF/MAPK activity

The aim of these experiments was to evaluate the effect of the inhibition of hydrogen sulfide physiological production using enzymes' specific inhibitors in various combinations. Subsequently, the MPF and MAPK activities during $3C_i$-suppressed oocyte maturation were analysed by the histone H1 and myelin basic protein (MBP) double assay.

No effect was observed of individual inhibitors expressed by MII achievement (Fig. 3A–C and Supplementary tables A1-A3), including for the double-combination of these inhibitors (Fig. 3D and Supplementary table A4). The triple-combination of hydrogen sulfide-releasing enzymes inhibitors significantly inhibited meiotic maturation in a dose-dependent manner (Fig. 4A and Supplementary table A5). For subsequent experiments, the most effective triple-combination ($3C_i$) of hydrogen sulfide-releasing enzymes inhibitors was used. Reversibly inhibited meiotic maturation and prolonged 72 h *in vitro* maturation showed deceleration, excluding a toxic effect of the used inhibitors. There were no statistically significant differences in oocyte maturation rate between the control oocytes and inhibited oocytes consequently treated

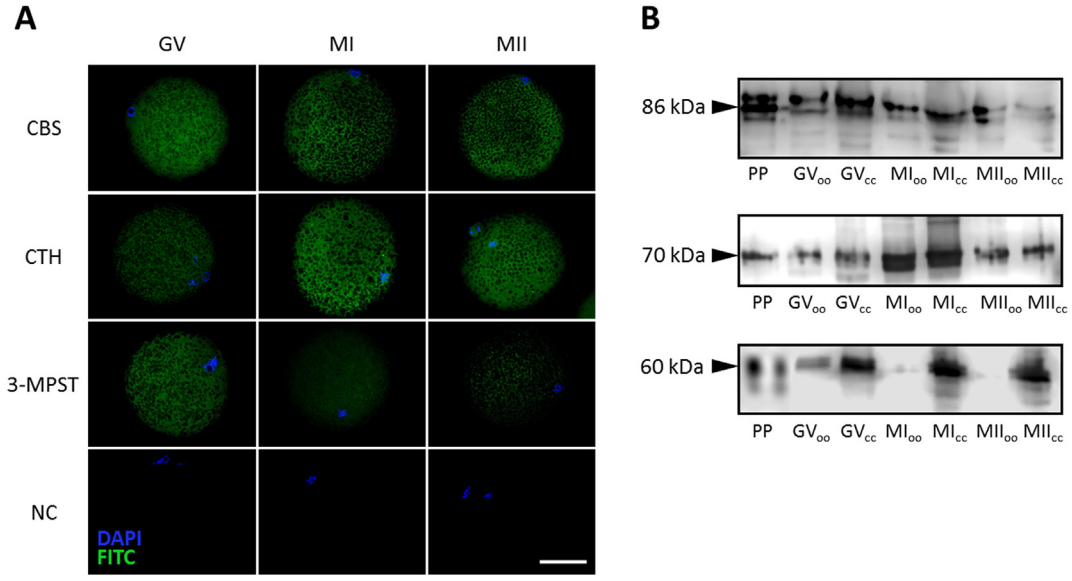
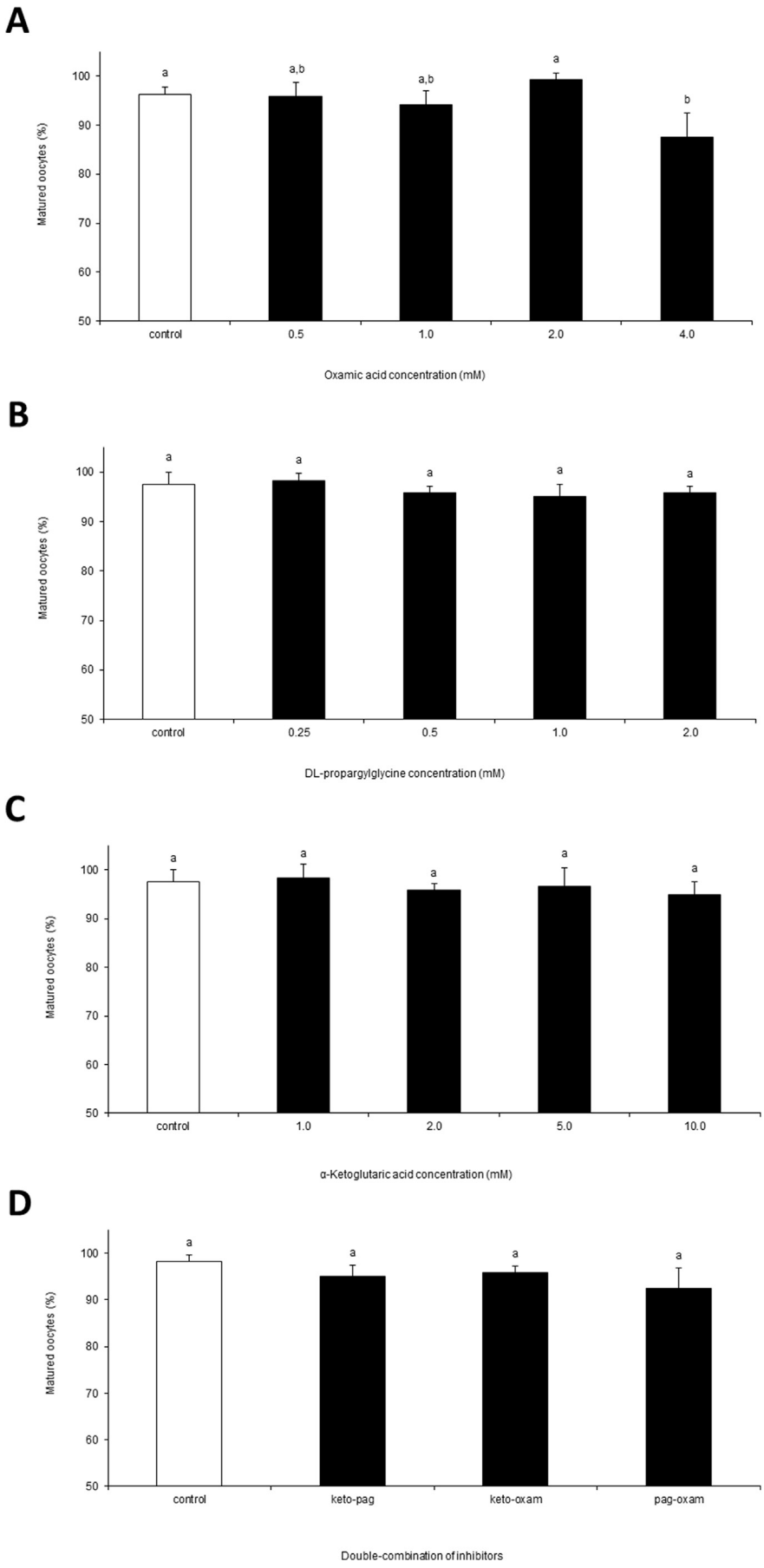


Fig. 2. Representative pictures of oocyte CBS, CTH and 3-MPST. The enzymes were immunocytochemically localised in immature GV, maturing MI and matured MII oocytes. B: The verification of enzyme presence by western blot analysis in oocytes and their cumulus cells. The immunocytochemical observation each enzyme was performed on 15 oocytes for each meiotic stage. Samples of 100 oocytes and their cumulus cells of each meiotic stage were used for western blot analysis. Scale bar = 50 μm.


A
Matured oocytes (%)
100
90
80
70
60
50
a
a,b
a,b
a
b
control
0.5
1.0
2.0
4.0
Oxamic acid concentration (mM)
B
Matured oocytes (%)
a
a
a
a
a
control
0.25
0.5
1.0
2.0
DL-propargylglycine concentration (mM)
C
Matured oocytes (%)
a
a
a
a
a
control
1.0
2.0
5.0
10.0
α-Ketoglutaric acid concentration (mM)
D
Matured oocytes (%)
a
a
a
a
control
keto-pag
keto-oxam
pag-oxam
Double-combination of inhibitors

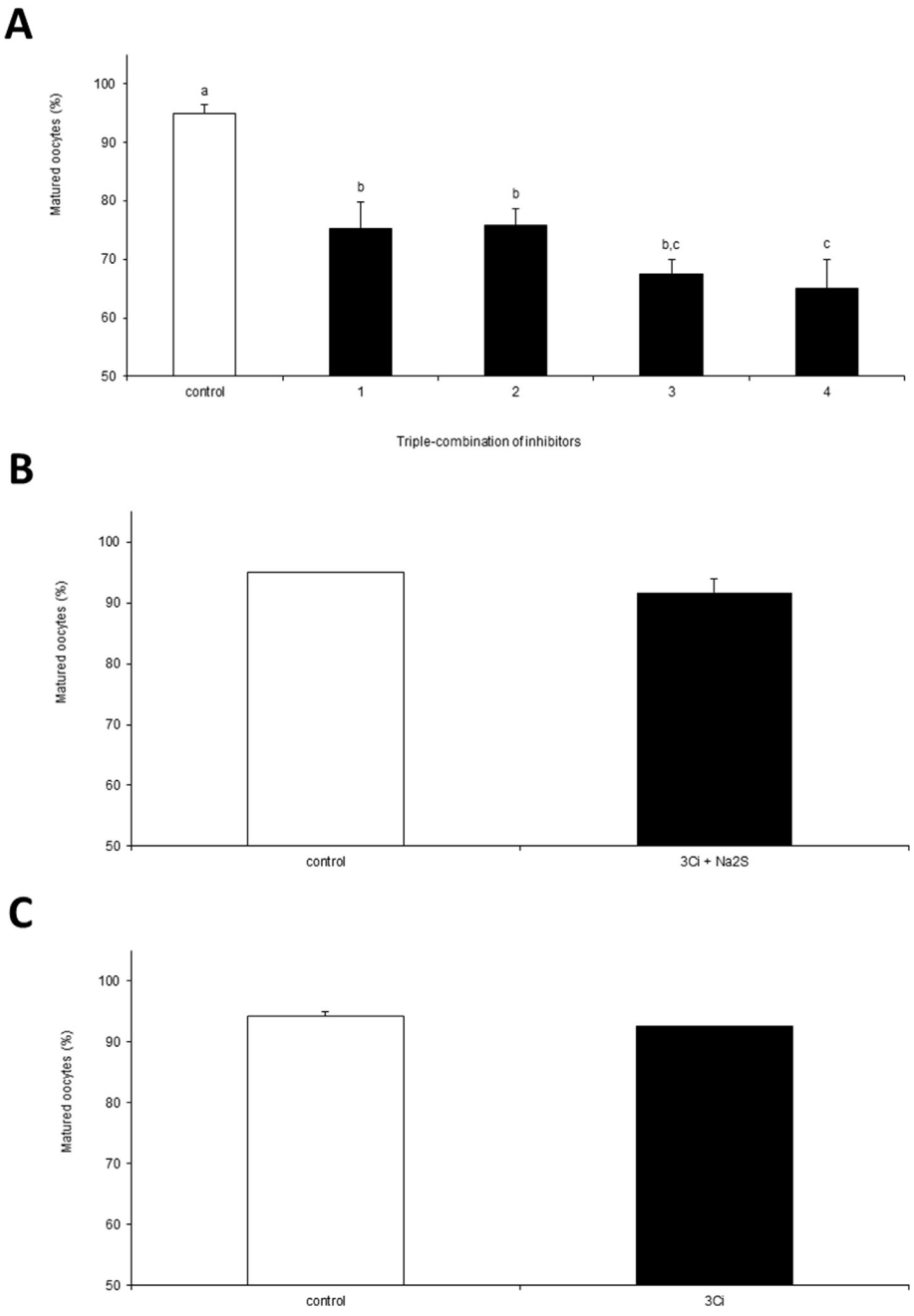


Fig. 4. Effect of restriction of hydrogen sulfide production on oocyte maturation. A: Proportion of matured oocytes and first polar body extrusion after treatment by triple-combination of inhibitors during 48 h *in vitro* maturation. Triple-combination: 1—0.25 mM oxamic acid, 0.25 mM DL-propargylglycine and 0.5 mM α-ketoglutaric acid; 2—0.5 mM oxamic acid, 0.5 mM DL-propargylglycine and 1 mM α-ketoglutaric acid; 3—1 mM oxamic acid, 1 mM DL-propargylglycine and 2.5 mM α-ketoglutaric acid; 4—2 mM oxamic acid, 2 mM DL-propargylglycine and 5 mM α-ketoglutaric acid ($3C_i$; considered as effective concentration in subsequent experiments). B: Reverse effect of hydrogen sulfide donor (300 μM $Na_2S.9H_2O$) on $3C_i$-suppressed oocyte maturation ($3C_i$ + Na_2S). C: Proportion of matured oocytes after prolonged 72 h cultivation under $3C_i$ treatment. Each experimental (A,B,C) group includes 120 in three independent experiments. Bars show the means of percentage ratio ± SEMs. Bars with different letters are significantly different. The GLM procedure followed by the Scheffe's test in SAS software was performed and experimental groups were tested each other ($P < 0.05$).

with $Na_2S.9H_2O$, a hydrogen sulfide donor (Fig. 4B and Supplementary table A6), and no significant differences between control and $3C_i$-inhibited oocytes after prolonged 72 h cultivation (Fig. 4C and Supplementary table A7).

The germinal vesicle breakdown (GVBD), metaphase I achievement and metaphase II achievement coupled with first polar body

Fig. 3. Effect of suppression of hydrogen sulfide-releasing enzymes and their double-combinations' usage during oocyte maturation. A,B,C: Proportion of matured oocytes and first polar body extrusion in control and inhibited groups of oocytes matured *in vitro* for 48 h with oxamic acid, DL-propargylglycine or α-ketoglutaric acid, an inhibitor of CBS, CTH and 3-MPST, respectively. D: Proportion of matured oocytes and first polar body extrusion after treatment by double-combination of inhibitors during 48 h *in vitro* maturation. Double-combination: oxam-pag – 2 mM oxamic acid and 2 mM DL-propargylglycine; oxam-keto – 2 mM oxamic acid and 5 mM α-ketoglutaric acid; pag-keto – 2 mM DL-propargylglycine and 5 mM α-ketoglutaric acid. Each experimental group includes 120 oocytes in three independent experiments. Bars show the means of percentage ratio ± SEMs. Bars with different letters are significantly different. The GLM procedure followed by Scheffe's test in SAS software was performed and experimental groups were tested against each other ($P < 0.05$).

were evaluated as markers of meiosis re-initiation, meiotic progress and the success of *in vitro* oocyte maturation under $3C_i$ treatment. There was observed significant slowdown of GVBD of 28.4–51.6% between 18 and 24 h cultivation (Fig. 5A), and further meiotic progress expressed by metaphase I (Fig. 5B) and metaphase II achievement (Fig. 5C) between 18 and 32 h of *in vitro* cultivation. See for more details Supplementary table A8.

The measurement of phosphorylated H1 and MBP, specific substrates of MPF and MAPK, respectively, show a decline in their kinase activity. The MPF activity significantly decreased after 24 h oocyte maturation (100 vs. 63.0 ± 6.7% for control and $3C_i$, respectively), with further decrease in activity following after 36 h of oocyte cultivation (94.5 ± 11.7 vs. 60.1 ± 4.8% for control and $3C_i$, respectively). In addition, significant suppression of MAPK activity after 24 h oocyte maturation occurred (100 vs. 63.8 ± 6.4% for control and $3C_i$, respectively). Data including representative autoradiographs is summarised in Fig. 5D and E.

3.4. Inhibition of hydrogen sulfide physiological production in porcine oocytes

The aim of the experiment was to reveal the physiological action of hydrogen sulfide-releasing enzymes, resulting in hydrogen sulfide production, in oocyte lysate. For proof of hydrogen sulfide-releasing enzymes' function, a triple-combination of their specific inhibitors ($3C_i$) in oocyte lysates and colorimetric measurement of hydrogen sulfide presence were performed.

Hydrogen sulfide production was not suppressed by $3C_i$ use in immature germinal vesicle (GV) oocytes. On the other hand, matured metaphase II (MII) oocytes were significantly inhibited in hydrogen sulfide production and hydrogen sulfide production decreased by 62.1%. Data is shown in Fig. 6.

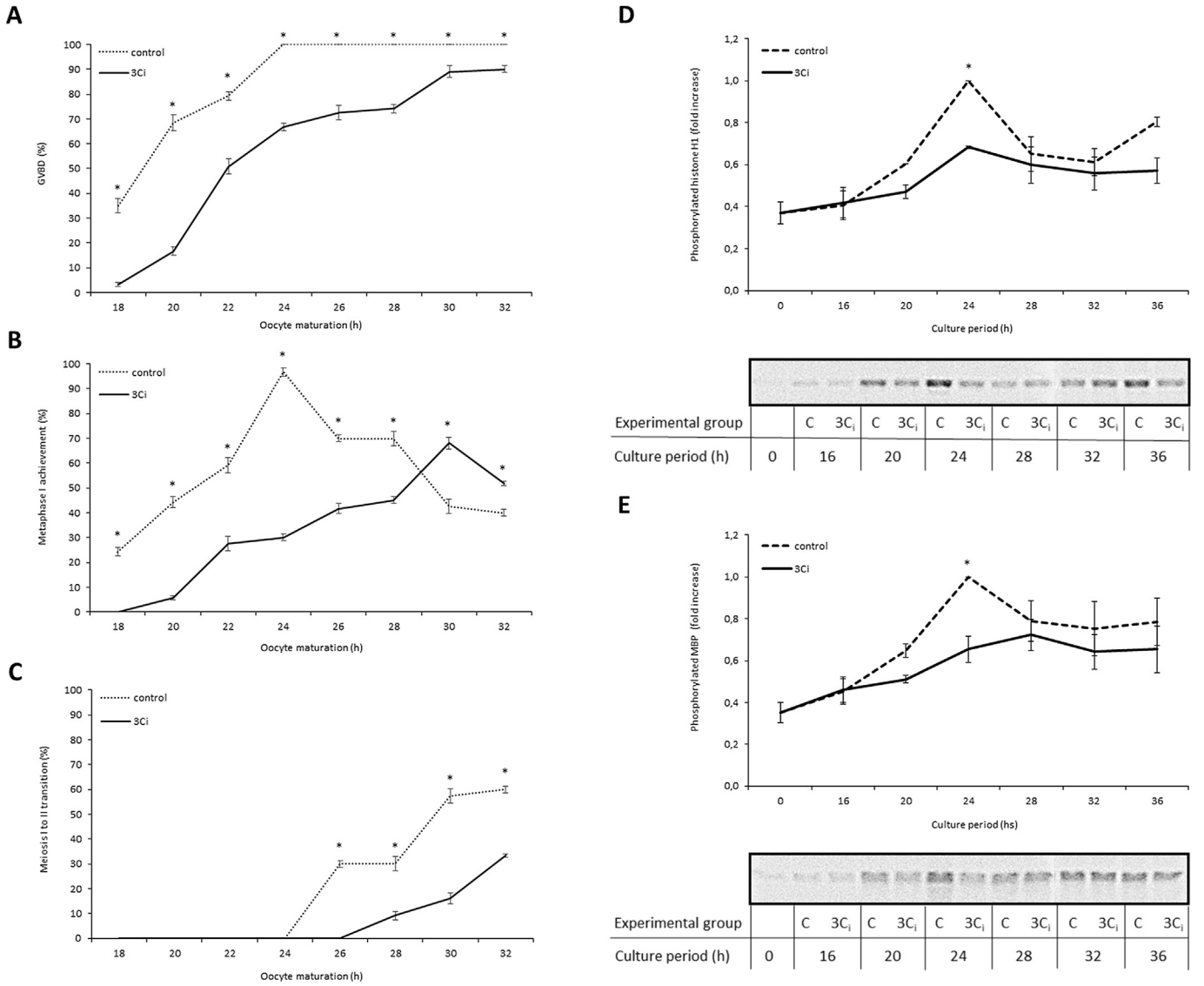


Fig. 5. Inhibition of hydrogen sulfide production by $3C_i$ and its effect on GVBD and the progress of meiotic maturation. A,B,C: Proportion of GVBD, MI achievement and meiosis I to II transition, respectively, during 32 h oocyte maturation were elucidated. Each experimental group includes 120 oocytes in all of the time points in three independent experiments. Points show the means of percentage ratio ± SEMs. Points with asterisk show significant difference between control and $3C_i$ groups ($P < 0.05$). D,E: Inhibition of hydrogen sulfide production by $3C_i$ and its effect on MPF and MAPK activities oocyte maturation. Signal quantifications and representative autoradiographs of phosphorylated histone H1 and MBP reflecting MPF and MAPK activity, respectively, during 32 h oocyte maturation. Each sample includes 15 oocytes in six independent experiments. Points show the means of signal intensity related to a control matured 24 h ± SEMs. Points with asterisk show significant difference between control and $3C_i$ groups ($P < 0.05$), using the GLM procedure followed by Scheffe's test in SAS software.

3.5. Effect of inhibition of hydrogen sulfide production on cumulus expansion of porcine cumulus-oocyte complexes

Hyaluronic acid (HA) spectrophotometric measurement was used to evaluate cumulus expansion. COCs were cultured for 24 and 48 h *in vitro* and treated by $3C_i$ of hydrogen sulfide-releasing enzymes. No statistically significant differences were observed in the retained and released HA after 24 h *in vitro* oocyte maturation. A significant reduction in HA retained in COCs (100 vs. 66.5 ± 6.5% for control and $3C_i$, respectively) was observed after 48 h of cultivation. On the other hand, a significant increase in released HA occurred after 48 h of cultivation (100 vs. 138.7 ± 16.1% for control and $3C_i$, respectively). Data is shown in Fig. 7.

4. Discussion

In this study, we described endogenous hydrogen sulfide production in mammalian oocytes and its physiological role during meiotic maturation. We demonstrated the presence of mRNA and proteins of CBS, CTH and 3-MPST, hydrogen sulfide-releasing enzymes, in porcine oocytes. Based on our observation, we deduce endogenous hydrogen sulfide ability in the regulation of oocyte meiosis and cumulus expansion. A previous study [20] describing mRNA CBS in mouse oocyte and cumulus cells has denoted hydrogen sulfide as a gasotransmitter involved in oocyte maturation. As such, endogenous production of hydrogen sulfide in oocytes is considered to be a potential regulator of events in oocyte and surrounding cumulus cells, but this role has been only indicated [20,21]. However, our study is the first one to demonstrate the presence and role of CTH and 3-MPST in mammalian oocytes and cumulus expansion. In addition to observing all the hydrogen sulfide-releasing enzymes in porcine oocytes and cumulus cells, we are the first to demonstrate endogenous hydrogen sulfide production in oocytes and its necessity for *in vitro* oocyte maturation.

The mRNAs and proteins of hydrogen sulfide-releasing enzymes detected in our experiments, suggest a specific role for these enzymes during oocyte growth and maturation. While mRNA CTH was present at an almost constant level for the whole of the growth and maturation phases, mRNA CBS presence in the oocyte is limited to the growing phase, mRNA in cumulus cells does not change during growth and increases during oocyte maturation. mRNA 3-MPST appears briefly in the oocyte shortly before meiosis reinitiation, suggesting 3-MPST is required for meiotic competence.

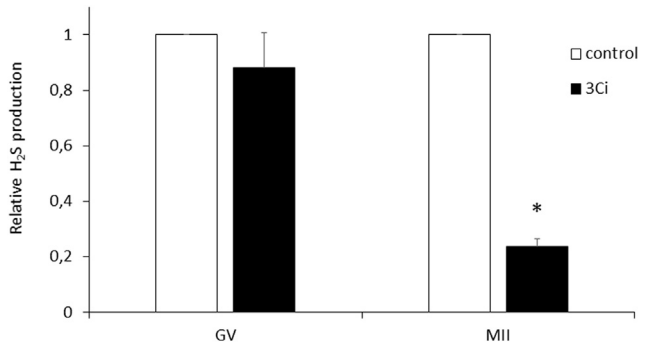


Fig. 6. Real hydrogen sulfide production in GV and matured MII oocytes verified with $3C_i$ treatment. The hydrogen sulfide production in $3C_i$-treated oocytes was related to GV and MII oocytes of control untreated groups following the calculation by standard curve. Each experimental group includes 150 oocytes in three independent experiments. Bars show the means of relative amount of hydrogen sulfide ± SEMs. A bar with asterisk indicates a significant difference between control and $3C_i$ groups for each maturation stage ($P < 0.05$), using the GLM procedure followed by Scheffe's test in SAS software.

Regardless of mRNA CBS and 3-MPST absence, both proteins are present in the oocyte during *in vitro* meiotic maturation. In addition to CBS and CTH, we detected the presence of 3-MPST in oocytes.

Although separate usage of hydrogen sulfide-releasing enzymes' inhibitors and their double-combinations did not have a significant effect on oocyte maturation, the effective triple-combination ($3C_i$) of all inhibitors suppressed meiotic maturation and affected cumulus expansion. We can assume that individual enzymes are capable of substituting for each other, and as such only the concurrent inhibition of all of them has a significant impact. A similar substitution of hydrogen sulfide-releasing enzymes is also presumed in somatic cells [28]. The observation of $3C_i$-suppressed GVBD and oocyte maturation is in agreement with our previous study describing maturation acceleration using Na_2S, an exogenous hydrogen sulfide donor [13].

Regarding the specific action of inhibitors, the Na_2S reverse effect on $3C_i$-suppressed oocyte maturation was tested. Moreover, the effect of inhibitors on physiological hydrogen sulfide production was proven by the significant fall in hydrogen sulfide content in the $3C_i$-treated oocyte lysate. Interestingly, a $3C_i$ effect was detected in matured MII, but not GV oocytes. We can speculate that immature no-cultured GV oocytes have a sufficient amount of hydrogen sulfide itself and enzymatic production is not necessary to keep its level up. Early maturing oocytes are able to utilise this hydrogen sulfide stock, and thus the matured oocyte is fully dependent on the action of hydrogen sulfide-releasing enzymes in a re-synthesised sufficient amount. A positive feedback loop may be due to the stimulation of CBS and CSE activity through S-sulfhydration, hydrogen sulfide-mediated post-translational protein modification [29,30]. Such a feedback loop may result from prompt hydrogen sulfide release in the early stage of oocyte maturation. Hydrogen sulfide-releasing enzymes in the immature oocyte can be present in sufficient amounts but with low physiological action. This assumption is in the accordance with our observed $3C_i$-suppressed GVBD during 18–32 h of *in vitro* cultivation as well as deceleration of following meiotic stages, where the presence of active hydrogen sulfide-releasing enzymes is already necessary and their inhibition is effective.

We discovered that meiotic maturation of $3C_i$-treated oocytes is not irreversibly inhibited but only suppressed. This is suggested by a further 24 h *in vitro* cultivation following 48 h maturation. This meiotic suppression was caused by slower MPF and MAPK activation at the beginning of oocyte cultivation. If the activation peaks of MPF and MAPK were damped, one can also note that they were not delayed, as might have been expected by observing the delay in meiotic progression. Conversely, we have described an accelerating effect of the hydrogen sulfide donor on MPF and MAPK activities [13].

The molecular mechanism of hydrogen sulfide's effect on MPF and MAPK regulation remains unknown, however S-sulfhydration changing the activity of various proteins [29,30] is one possible explanation. Of these, ion channels involved in the regulation of mammalian oocyte maturation [summarised in Ref. [31]] are post-translationally modified by hydrogen sulfide, as well as various enzymes with catalytic ability [29,32,33]. As such, we can assume regulation of MPF and MAPK activities and/or their up-stream kinases or phosphatases. S-sulfhydration and activation of MEK1, a direct MAPK regulator, is known to occur in somatic cells [34], and it can be presumed in oocytes too. Another possible explanation is cAMP/PKA [35] and PI3K/Akt [36] regulation by hydrogen sulfide, signal pathways essential for oocyte maturation [37,38]. Phosphatases activities can also be targeted by hydrogen sulfide [39,40].

One might also mention that S-sulfhydration has been reported to potentially modulate the production of other gasotransmitters such as nitric oxide and carbon monoxide. S-sulfhydration of

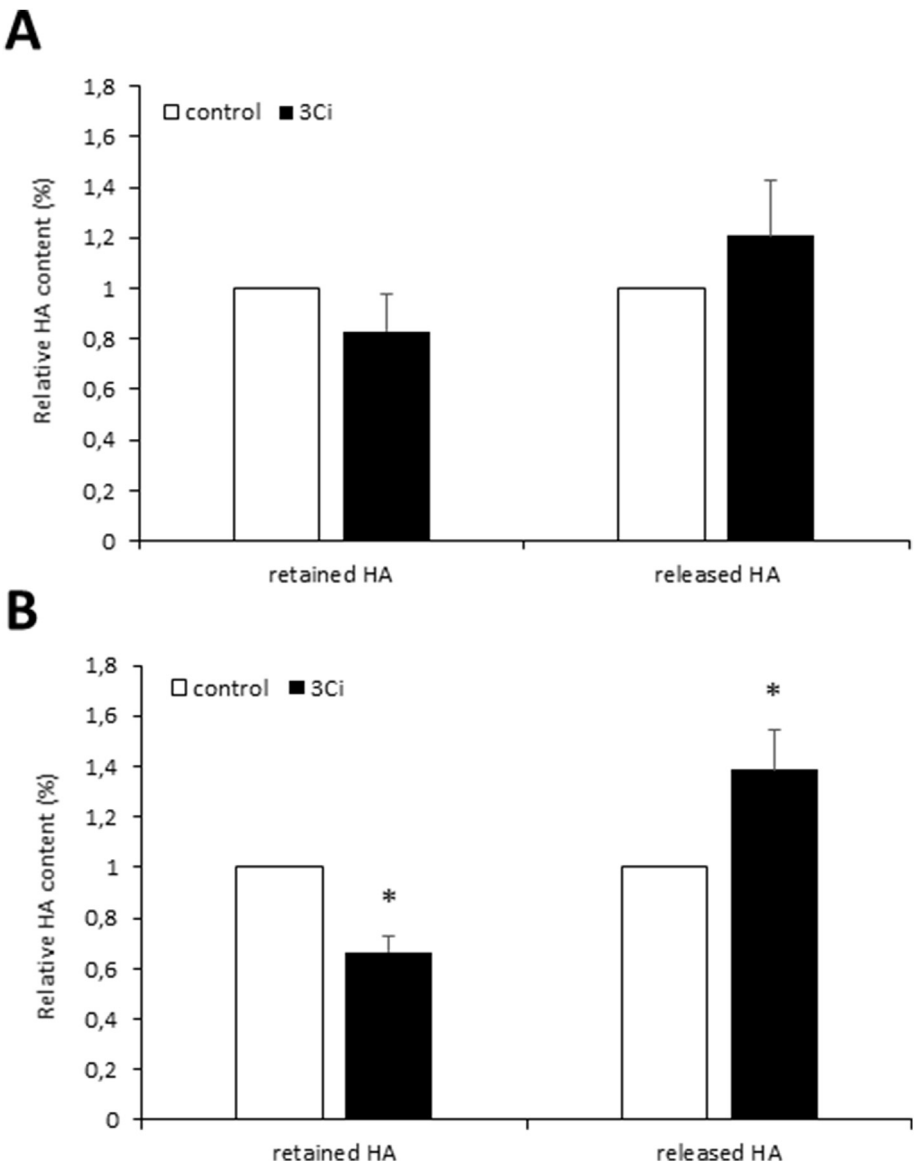


Fig. 7. Effect of $3C_i$-inhibited production of hydrogen sulfide on HA synthesis during cumulus expansion. A,B: HA retained in COCs and HA released into the culture medium were measured after 24 and 48 h of cultivation, respectively, as markers of cumulus expansion. The HA retained and HA released in $3C_i$-treated COCs was related to MI and MII oocytes of control untreated groups following the calculation by standard curve. Each sample includes 25 oocytes in six independent experiments. Bars show the means of relative amount of HA ± SEMs. A bar with asterisk indicates a significant difference between control and $3C_i$ ($P < 0.05$), using the GLM procedure followed by Scheffe's test in SAS software.

endothelial nitric oxide synthase and increases in its activity have been described [33]. In addition to S-sulfhydration, hydrogen sulfide acts via interactions with other gasotransmitters in somatic cells [41,42]. Although gasotransmitters' cross-talk are not understood in oocytes, we cannot discard nitric oxide down-regulation, and as such indirect hydrogen sulfide influence on oocyte maturation key signal pathways, such as the already mentioned cAMP/PKA or cGMP/PKG, known targets of nitric oxide [43,44].

In addition to their roles during *in vitro* oocyte maturation, gasotransmitters may impact on hypothalamic-pituitary-gonadal axis regulation *in vivo,* as already described [45–47]. Hydrogen sulfide-directed functions of pituitary cells are known [48]. As such, the overall effects of hydrogen sulfide resulting in regulation of gonadotropin releasing hormone secretion and/or modulation of gonadal activity in hormone secretion can be expected. Clearly, the observed hydrogen sulfide effect on *in vitro* maturing oocytes is only part of hydrogen sulfide's many abilities, including gasotransmitter interactions. Nevertheless, our context was not dependent upon such an effect, since experiments were performed independently upon the neuro-endocrine pathway.

Our observation of mRNA and proteins of CBS, CTH and 3-MPST in cumulus cells indicates that hydrogen sulfide has a regulating effect in cumuli of cumulus-oocyte complexes. This presumption has been improved by the inhibition of hydrogen sulfide production and its effect on cumulus expansion. In our previous study [13], we have demonstrated the inhibitory effect of an exogenous hydrogen sulfide donor on HA production in the extracellular matrix of expanded cumuli. In this study, the inhibition of hydrogen sulfide-releasing enzymes does not influence HA production after 24 h *in vitro* cultivation of COCs. After 48 h cultivation, decreasing HA retention in COCs and a boost in HA being released into the culture medium occurred. The suppression in HA production can be associated with observed oocyte maturation failure. In addition to mentioned impact of cumulus expansion for oocyte maturation, the

protective effect of HA on cell viability [49] and its lack could be responsible for a decrease in oocyte quality through pro-apoptotic signal pathways present in oocytes [50].

This study gives the first description of all the hydrogen sulfide-releasing enzymes in mammalian oocytes and confirmation of their physiological action consisting of hydrogen sulfide release. The requirement of hydrogen sulfide in MPF and MAPK-induced oocyte maturation at particular times has been observed. Moreover, HA production by expanded cumuli seems to be regulated by endogenously produced hydrogen sulfide. However, further experiments testing the exact mechanism of hydrogen sulfide action are required to fully understand gasotransmitter signal pathways in mammalian oocytes.

Conflict of interest statement

The authors have no conflicts of interest to declare.

Acknowledgements

This work was supported by the National Agency for Agricultural Research (NAZV QJ1510138), the Czech Ministry of Agriculture (MZeRO 0714), the Czech Ministry of Education, Youth and Sports of the Czech Republic (Institutional Developing Project – the Long-term Ph.D. Intership Foundation) and the Czech University of Life Sciences in Prague (CIGA 20132035). We would like to thank the Institute of Experimental Botany of the Academy of Sciences of the Czech Republic for their technical support and Mr. Brian Kavalir for his editorial assistance with this manuscript.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.niox.2015.09.007.

References

[1] P.M. Wassarman, The mammalian ovum, in: E. Knobil, J. Neill (Eds.), The Physiology of Reproduction, Raven Press, New York, 1988, pp. 69–102.
[2] R. Yanagimachi, Mammalian fertilization, in: E. Knobil, J. Neill (Eds.), The Physiology of Reproduction, Raven Press, New York, 1988, pp. 230–278.
[3] N. Dekel, Y. Hillensjo, P.F. Kraicer, Maturation effects of gonadotropins on the cumulus-oocyte complex of the rat, Biol. Reprod. 20 (1979) 191–197.
[4] J.J. Eppig, FSH stimulates hyaluronic acid synthesis by oocyte-cumulus cell complexes from mouse preovulatory follicles, Nature 281 (1979) 483–484.
[5] T. Nakayama, M. Inoue, E. Sato, Effect of oocytectomy on glycosaminoglycan composition during cumulus expansion of porcine cumulus-oocyte complexes cultured *in vitro*, Biol. Reprod. 55 (1996) 1299–1304.
[6] A. Salustri, A. Camaioni, E. Tirone, C. D'Alessandris, Italian J. Anat. Embryol. 100 (Suppl. 1) (1995) 479–484.
[7] N. Dekel, T.S. Lawrence, N.B. Gilula, W. Beers, Modulation of cell-to-cell communication in the cumulus-oocyte complex and the regulation of oocyte maturation by LH, Dev. Biol. 86 (1981) 356–362.
[8] J. Tornell, M. Brannstorm, T. Hillensjo, Different effects of cyclic-nucleotide derivates upon the rat oocyte–cumulus complex *in vitro*, Acta Physiol. Scand. 122 (1984) 507–513.
[9] M. Yokko, T. Shimizu, N. Kimura, W.A. Tunjung, H. Matsumoto, H. Abe, H. Sasada, H. Rodriguez-Martinez, E. Sato, Role of the hyaluronan receptor CD44 during porcine oocyte maturation, J. Reprod. Dev. 53 (2007) 263–270.
[10] M. Yokoo, N. Kimura, E. Sato, Induction of oocyte maturation by hyaluronan-CD44 interaction in pigs, J. Reprod. Dev. 56 (2010) 15–19.
[11] M. Kubelka, J. Motlik, R.M. Schultz, A. Pavlok, Butyrolactone I reversibly inhibits meiotic maturation of bovine oocytes, without influencing chromosome condensation activity, Biol. Reprod. 62 (2000) 292–302.
[12] Q.Y. Sun, Q. Lu, H. Breitbart, D.Y. Chen, CAMP inhibits mitogen-activated protein (MAP) kinase activation and resumption of meiosis, but exerts no effects after spontaneous germinal vesicle breakdown (GVBD) in mouse oocytes, Reprod. Fertil. Dev. 11 (1999) 81–86.
[13] J. Nevoral, A. Gelaude, J.F. Bodart, V. Kucerova-Chrpova, M. Sedmikova, T. Krejcova, T. Kolbabova, M. Dvorakova, A. Vyskocilova, I. Weingartova, L. Krivohlavkova, T. Zalmanova, F. Jilek, Dual effect of hydrogen sulfide donor on meiosis and cumulus expansion of porcine cumulus-oocyte complexes, PLoS One 9 (2014) e99613.
[14] T. Krejcova, M. Smelcova, J. Petr, J.F. Bodart, M. Sedmikova, J. Nevoral, M. Dvorakova, A. Vyskocilova, I. Weingartova, V. Kucerova-Chrpova, E. Chmelikova, L. Tumova, F. Jilek, Hydrogen sulfide donor protects porcine oocytes against aging and improves the developmental potential of aged porcine oocytes, PLoS One 10 (2015) e0116964.
[15] J. Nevoral, M. Orsak, P. Klein, J. Petr, M. Dvorakova, I. Weingartova, A. Vyskocilova, K. Zamostna, T. Krejcova, F. Jilek, Cumulus cell expansion, a role in oocyte biology and its measurement: a review, Sci. Agric. Bohem. 45 (2014) 13–26.
[16] H. Kimura, The physiological role of hydrogen sulfide and beyond, Nitric Oxide 41 (2014) 4–10.
[17] Q. Li, J.R. Lancaster Jr., Chemical foundations of hydrogen sulfide biology, Nitric Oxide 35 (2013) 21–34.
[18] R. Wang, Signaling pathways for the vascular effects of hydrogen sulphide, Curr. Opin. Nephrol. Hypertens. 20 (2011) 107–112.
[19] N. Shibuya, M. Tanaka, M. Yoshida, Y. Ogasa-Wara, T. Togawa, K. Ishii, H. Kimura, 3-Mercaptopyruvate sulfurtransferase produces hydrogen sulfide and bound sulfane sulfur in the brain, Antioxid. Redox. Signal. 11 (2009) 703–714.
[20] R. Liang, W.D. Yu, J.B. Du, L.J. Yang, M. Shang, J.Z. Guo, Localization of cystathionine β synthase in mice ovaries and its expression profile during follicular development, Chin. Med. J. 119 (2006) 1877–1883.
[21] R. Liang, W.D. Yu, J.B. Du, L.J. Yang, J.J. Yang, X. Jian, M. Shang, J.Z. Guo, Cystathionine β synthase participates in murine oocytes maturatione mediated by homocysteine, Reprod. Toxicol. 24 (2007) 89–96.
[22] J. Motlik, J. Fulka, Breakdown of germinal vesicle in pig oocytes *in vivo* and *in vitro*, J. Exp. Zool. 198 (1976) 155–162.
[23] K.J. Livak, T.D. Schmittgen, Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ Method, Methods 25 (2001) 402–408.
[24] E.W. Kuijk, L. du Puy, H.T. van Tol, H.P. Haagsman, B. Colenbrander, B.A. Roelen, Validation of reference genes for quantitative RT-PCR studies in porcine oocytes and preimplantation embryos, BMC Dev. Biol. 7 (2007) 58.
[25] Y.J. Yi, M. Sutovsky, W.H. Song, P. Sutovsky, Protein deubiquitination during oocyte maturation influences sperm function during fertilisation, anti-polyspermy defense and embryo development, Reprod. Fertil. Dev. (2014), http://dx.doi.org/10.1071/RD14012.
[26] L. Tůmová, J. Petr, T. Žalmanová, E. Chmelíková, T. Kott, H. Tichovská, V. Kučerová-Chrpová, K. Hošková, F. Jílek, Calcineurin expression and localisation during porcine oocyte growth and meiotic maturation, Anim. Reprod. Sci. 141 (2013) 154–163.
[27] K. Abe, H. Kimura, The possible role of hydrogen sulfide as an endogenous neuromodulator, J. Neurosci. 16 (1996) 1066–1071.
[28] R. Sanokawa-Akakura, E.A. Ostrakhovitch, S. Akakura, S. Goodwin, S. Tabibzadeh, A H_2S-Nampt dependent energetic circuit is critical to survival and cytoprotection from damage in cancer cells, PLoS One 9 (2014) e108537.
[29] A.K. Mustafa, M.M. Gadalla, N. Sen, S. Kim, W. Mu, S.K. Gazi, R.K. Barrow, G. Yang, R. Wang, S.H. Snyder, H_2S signals through protein S-sulfhydration, Sci. Signal. 2 (2009) ra72.
[30] N. Sen, B.D. Paul, M.M. Gadalla, A.K. Mustafa, T. Sen, R. Xu, S. Kim, S.H. Snyder, Hydrogen sulfide-linked sulfhydration of NF-κB mediates its antiapoptotic actions, Mol. Cell 45 (2012) 13–24.
[31] E. Tosti, R. Boni, A. Gallo, F. Silvestre, Ion currents modulating oocyte maturation in animals, Syst. Biol. Reprod. Med. 59 (2013) 61–68.
[32] B.D. Paul, S.H. Snyder, H_2S signalling through protein sulfhydration and beyond, Nat. Rev. Mol. Cell Biol. 13 (2012) 499–507.
[33] Z. Altaany, Y. Ju, G. Yang, R. Wang, The coordination of S-sulfhydration, S-nitrosylation, and phosphorylation of endothelial nitric oxide synthase by hydrogen sulphide, Sci. Signal 7 (2014) ra87.
[34] K. Zhao, Y. Ju, S. Li, Z. Altaany, R. Wang, G. Yang, S-sulfhydration of MEK1 leads to PARP-1 activation and DNA damage repair, EMBO Rep. 15 (2014) 792–800.
[35] Y.F. Njie-Mbye, M. Kulkarni, C.A. Opere, S.E. Ohia, Mechanism of action of hydrogen sulfide on cyclic AMP formatrion in rat retinal pigment epithelial cells, Exp. Eye Res. 98 (2012) 16–22.
[36] Y. Hu, X. Chen, T.T. Pan, K.L. Neo, S.W. Lee, E.S. Khin, P.K. Moore, J.S. Bian, Cardioprotection induced by hydrogen sulfide preconditioning involves activation of ERK and PI3K/Akt pathways, Pflugers Arch. 455 (2008) 607–616.
[37] M. Kovo, M. Kandli-Cohen, M. Ben-Haim, D. Galiani, D.W. Carr, N. Dekel, An active protein kinase A (PKA) is involved in meiotic arrest of rat growing oocytes, Reproduction 132 (2006) 33–43.
[38] J. Kalous, M. Kubelka, P. Solc, A. Susor, J. Motlík, AKT (protein kinase B) is implicated in meiotic maturation of porcine oocytes, Reproduction 138 (2009) 645–654.
[39] G. Buhrman, B. Parker, J. Sohn, J. Rudolph, C. Mattos, Structural mechanism of oxidative regulation of the phosphatase Cdc25B via an intramolecular disulfide bond, Biochemistry 44 (2005) 5307–5316.
[40] E. Viry, A. Anwar, G. Kirsch, C. Jacob, M. Diederich, D. Bagrel, Antiproliferative effect of natural tetrasulfides in human breast cancer cells is mediated through the inhibition of the cell division cycle 25 phosphatases, Int. J. Oncol. 38 (2011) 1103–1111.
[41] C.L. Heine, R. Schmidt, K. Geckl, A. Schrammel, B. Gesslbauer, K. Schmidt, B. Mayer, A.C. Gorren, Selective irreversible inhibition of neuronal and inducible nitric-oxide synthase in the combined presence of hydrogen sulfide and nitric oxide, J. Biol. Chem. (2015) pii: jbc.M115.660316, ahead of print.
[42] G. Yuan, C. Vasavda, Y.J. Peng, V.V. Makarenko, G. Raghuraman, J. Nanduri,

M.M. Gadalla, G.L. Semenza, G.K. Kumar, S.H. Snyder, N.R. Prabhakar, Protein kinase G-regulated production of H_2S governs oxygen sensing, Sci. Signal 8 (2015) ra37.
[43] S. Bu, H. Xie, Y. Tao, J. Wang, G. Xia, Nitric oxide influences the maturation of cumulus cell-enclosed mouse oocytes cultured in spontaneous maturation medium and hypoxanthine-supplemented medium through different signaling pathways, Mol. Cell. Endocrinol. 223 (2004) 85–93.
[44] J. Petr, R. Rajmon, E. Chmelíková, M. Tománek, V. Lánská, M. Pribánová, F. Jílek, Nitric-oxide-dependent activation of pig oocytes: the role of the cGMP-signalling pathway, Zygote 14 (2006) 9–16.
[45] C.A. Lamar, V.B. Mahesh, D.W. Brann, Regulation of gonadotrophin-releasing hormone (GnRH) secretion by heme molecules: a regulatory role for carbon monoxide? Endocrinology 137 (1996) 790–793.
[46] C. Mancuso, P. Navarra, P. Preziosi, Roles of nitric oxide, carbon monoxide, and hydrogen sulfide in the regulation of the hypothalamic–pituitary–adrenal axis, J. Neurochem. 113 (2010) 563–575.
[47] V. Prevot, D. Croix, C.M. Rialas, P. Poulain, G.L. Fricchione, G.B. Stefano, J.-C. Beauvillain, Estradiol coupling to endothelial nitric oxide stimulates gonadotropin-releasing hormone release from rat median eminence via a membrane receptor, Endocrinology 140 (1999) 652–659.
[48] G.F. Sitdikova, T.M. Weiger, A. Hermann, Hydrogen sulfide increases calcium-activated potassium (BK) channel activity of rat pituitary tumor cells, Pflügers Arch. 459 (2010) 389–397.
[49] W.A. Tunjung, M. Yokoo, Y. Hoshino, Y. Miyake, A. Kadowaki, E. Sato, Effect of hyaluronan to inhibit caspase activation in porcine granulosa, Biochem. Biophys. Res. Commun. 382 (2009) 160–164.
[50] M. Sedmíková, J. Petr, A. Dörflerová, J. Nevoral, B. Novotná, T. Krejčová, E. Chmelíková, L. Tůmová, Inhibition of c-Jun N-terminal kinase (JNK) suppresses porcine oocyte ageing *in vitro*, Czech J. Anim. Sci. 58 (2013) 535–545.

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

Markéta Dvořáková[1], Ivona Heroutová[1], David Němeček[1], Kateřina Adámková[1], Tereza Krejčová[1], Jan Nevoral[1,2], Veronika Kučerová Chrpová[1], Jaroslav Petr[3] and Markéta Sedmíková[1]

[1] Department of Veterinary Sciences, Czech University of Life Sciences, Prague, Czech Republic
[2] Biomedical center, Faculty of Medicine in Pilsen, Charles University in Prague, Plzen, Czech Republic
[3] Institute of Animal Science, Prague, Czech Republic

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.

Submitted 8 May 2016
Accepted 2 July 2016
Published 4 August 2016

Corresponding author
Markéta Dvořáková,
dvorakova2@af.czu.cz

Academic editor
Xiang-Jiao Yang

Additional Information and Declarations can be found on page 9

DOI **10.7717/peerj.2280**

Subjects Biotechnology, Developmental Biology, Veterinary Medicine
Keywords Antioxidant, S-allyl cysteine, Garlic, Oocyte, Pigs

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.

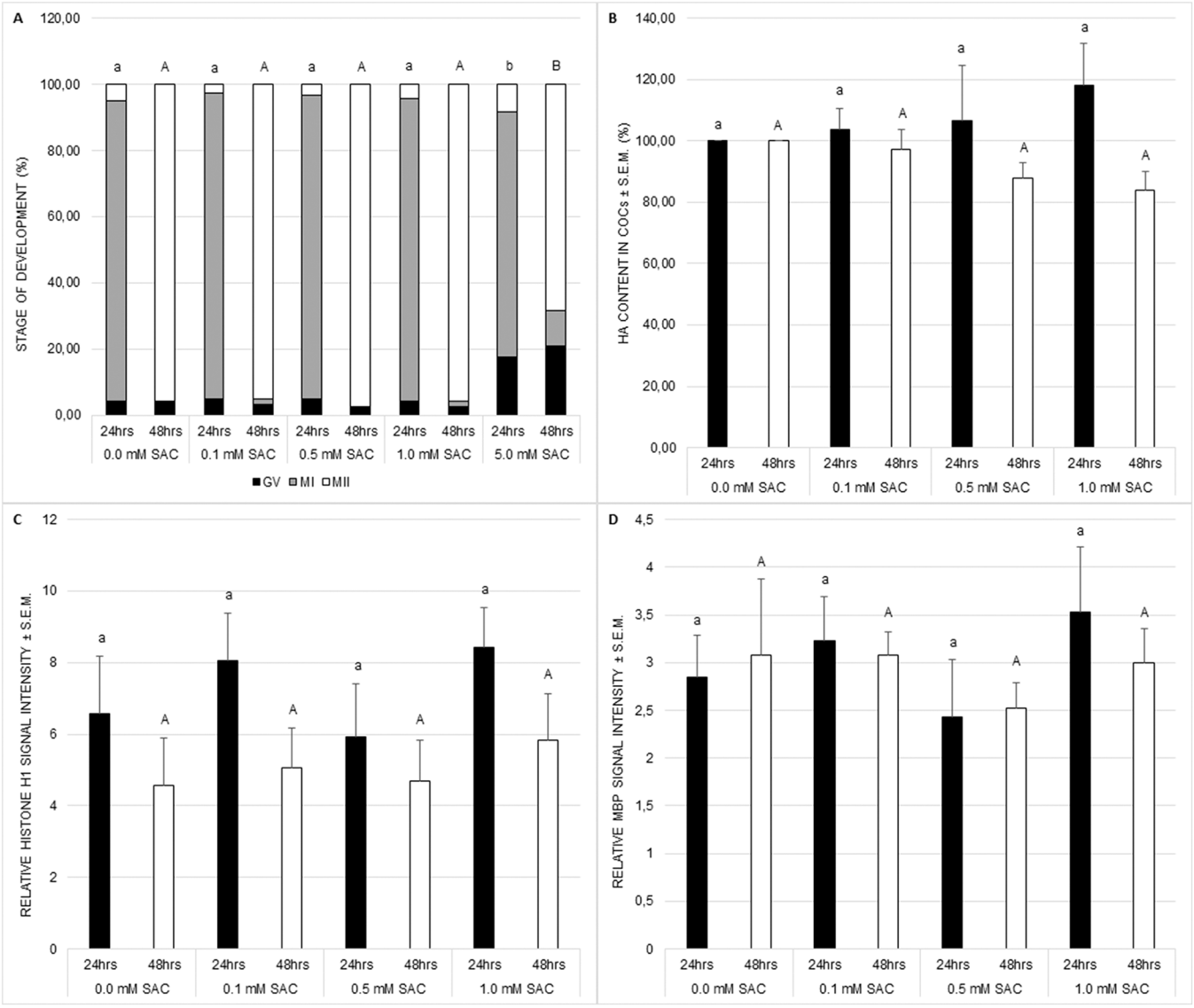


Figure 1 Effects of various SAC concentrations on porcine oocyte meiotic maturation after 24 and 48 h of cultivation. (A) Effects of SAC on nuclear maturation. GV–oocytes in the germinal vesicle stage, MI–oocytes in metaphase I, and MII–oocytes in metaphase II. Statistically significant differences between 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).

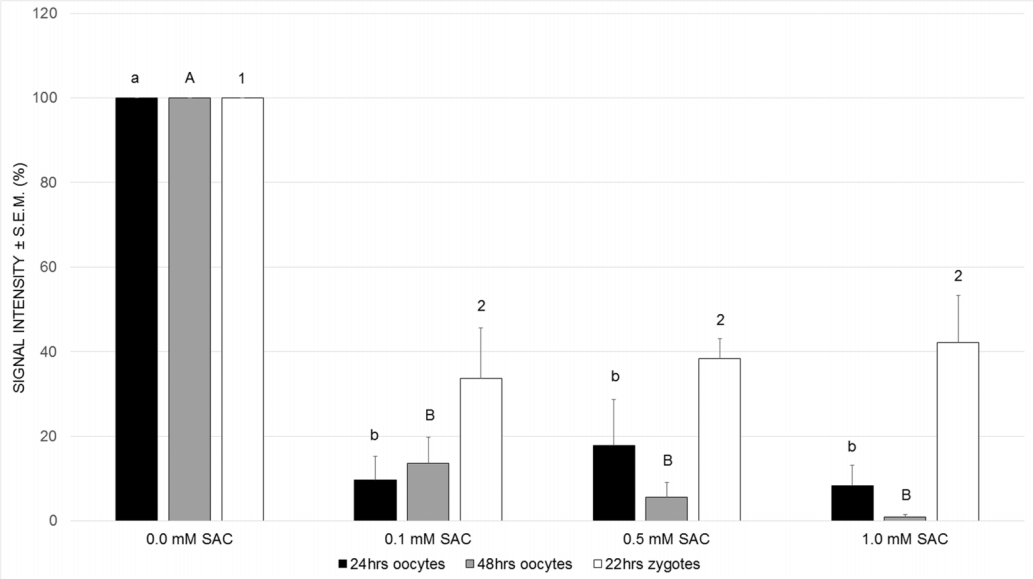


Figure 2 Effects of SAC on ROS production in porcine oocytes after 24 and 48 h of cultivation, and in zygotes after 22 h of cultivation. Statistically significant differences between ROS levels from various SAC concentrations are indicated by different superscripts: a, b–differences between ROS levels 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

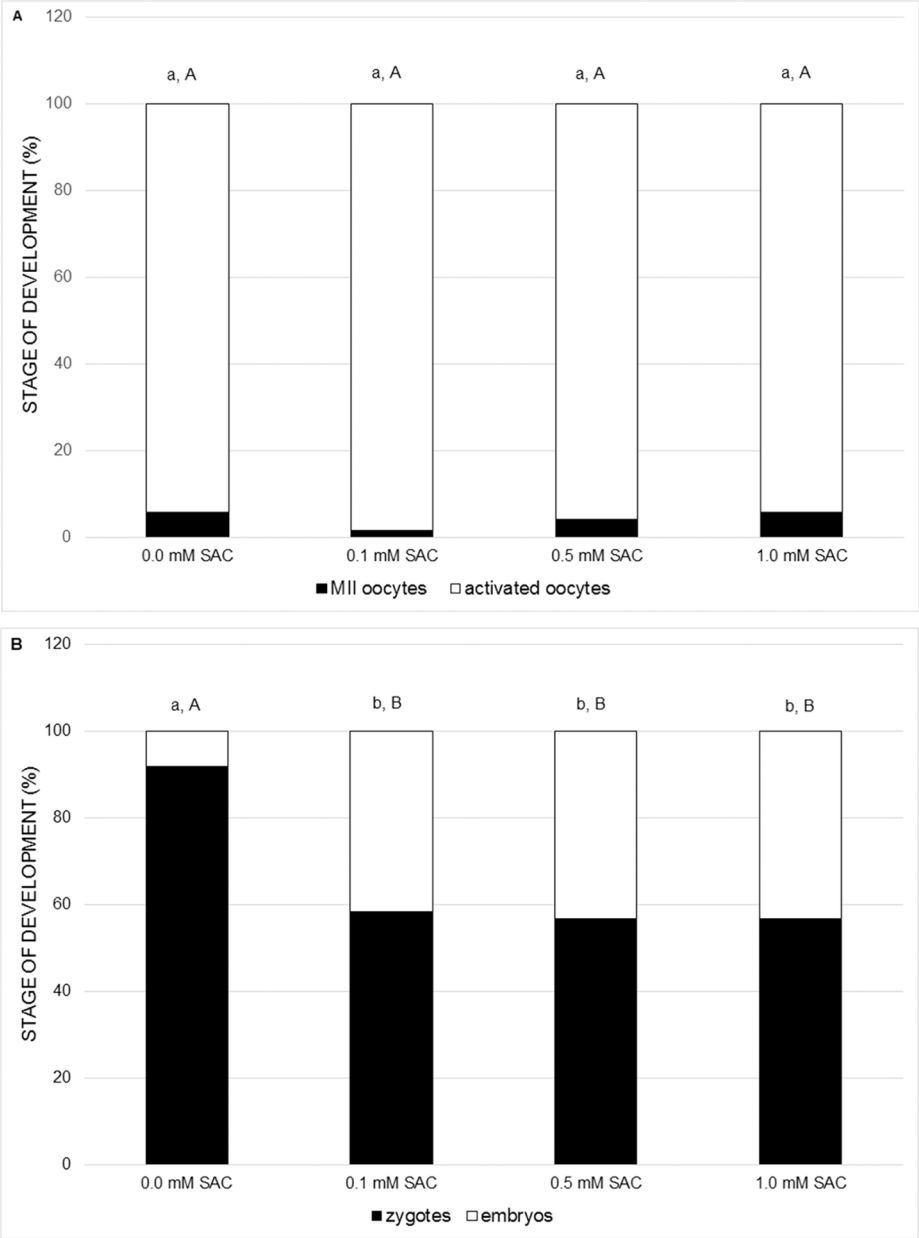


Figure 3 Effects of various SAC concentrations on parthenogenic activation of porcine oocytes. (A) Effects of SAC on the activating potential of parthenogenetically activated oocytes after 22 h of cultivation. MII oocytes–oocytes in metaphase II, activated oocytes–zygotes with one or two pronuclei and 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

Funding

This work was supported by the Ministry of Agriculture of the Czech Republic (NAZV–Project No. QJ1510138; MZeRO 0714) and by Internal Grant Agency of the Czech University of Life Sciences Prague (CIGA) (Project No. CZU20142049). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Grant Disclosures

The following grant information was disclosed by the authors:
Ministry of Agriculture of the Czech Republic: NAZV–QJ1510138; MZeRO 0714.
Czech University of Life Sciences Prague (CIGA): CZU20142049.

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.

REFERENCES

Agarwal A, Saleh RA, Bedaiwy MA. 2003. Role of reactive oxygen species in the pathophysiology of human reproduction. *Fertility and Sterility* **79(4):**829–843 DOI 10.1016/S0015-0282(02)04948-8.

Alvarez GM, Morado SA, Soto MP, Dalvit GC, Cetica PD. 2015. The control of reactive oxygen species influences porcine oocyte *in vitro* maturation. *Reproduction in Domestic Animals* **50(2):**200–205 DOI 10.1111/rda.12469.

Ambruosi B, Filioli Uranio M, Sardanelli AM, Pocar P, Martino NA, Paternoster MS, Amati F, Dell'Aquila ME. 2011. In vitro acute exposure to DEHP affects oocyte meiotic maturation, energy and oxidative stress parameters in a large animal model. *PLoS ONE* **6(11):**e27452 DOI 10.1371/journal.pone.0027452.

Choi W-J, Banerjee J, Falcone T, Bena J, Agarwal A, Sharma RK. 2007. Oxidative stress and tumor necrosis factor–α–induced alterations in metaphase II mouse oocyte spindle structure. *Fertility and Sterility* **88(4):**1220–1231 DOI 10.1016/j.fertnstert.2007.02.067.

Chung LY. 2006. The antioxidant properties of garlic compounds: allyl cysteine, alliin, allicin, and allyl disulfide. *Journal of Medicinal Food* **9(2):**205–213 DOI 10.1089/jmf.2006.9.205.

Colín-González AL, Ali SF, Túnez I, Santamaría A. 2015. On the antioxidant, neuroprotective and anti-inflammatory properties of S-allyl cysteine: an update. *Neurochemistry International* **89**:83–91 DOI 10.1016/j.neuint.2015.06.011.

Dion ME, Agler M, Milner JA. 1997. S-allyl cysteine inhibits nitrosomorpholine formation and bioactivation. *Nutrition and Cancer* **28(1):**1–6 DOI 10.1080/01635589709514545.

Giorgi VSI, Da Broi MG, Paz CCP, Ferriani RA, Navarro PA. 2015. N-acetyl-cysteine and L-carnitine prevent meiotic oocyte damage induced by follicular fluid from infertile

women with mild endometriosis. *Reproductive Sciences* **23(3):**342–351 DOI 10.1177/1933719115602772.

Hashimoto S, Minami N, Yamada M, Imai H. 2000. Excessive concentration of glucose during in vitro maturation impairs the developmental competence of bovine oocytes after in vitro fertilization: relevance to intracellular reactive oxygen species and glutathione contents. *Molecular Reproduction and Development* **56(4):**520–526 DOI 10.1002/1098-2795(200008)56:4<520::AID-MRD10>3.0.CO;2-0.

Hsu CC, Huang CN, Hung YC, Yin MC. 2004. Five cysteine-containing compounds have antioxidative activity in Balb/cA mice. *Journal of Nutrition* **134(1):**149–152.

Jablonka-Shariff A, Olson LM. 1998. The role of nitric oxide in oocyte meiotic maturation and ovulation: meiotic abnormalities of endothelial nitric oxide synthase knock-out mouse oocytes. *Endocrinology* **139(6):**2944–2954 DOI 10.1210/endo.139.6.6054.

Jiao G-Z, Cao X-Y, Cui W, Lian H-Y, Miao Y-L, Wu X-F, Han D, Tan J-H. 2013. Developmental potential of prepubertal mouse oocytes is compromised due mainly to their impaired synthesis of glutathione. *PLoS ONE* **8(3):**e58018 DOI 10.1371/journal.pone.0058018.

Jílek F, Hüttelová R, Petr J, Holubová M, Rozinek J. 2001. Activation of pig oocytes using calcium ionophore: effect of the protein kinase inhibitor 6-dimethyl aminopurine. *Reproduction in Domestic Animals* **36(3–4):**139–145 DOI 10.1046/j.1439-0531.2001.00257.x.

Kamoun P. 2004. Endogenous production of hydrogen sulfide in mammals. *Amino Acids* **26(3):**243–254 DOI 10.1007/s00726-004-0072-x.

Kohen R, Nyska A. 2002. Oxidation of biological systems: oxidative stress phenomena, antioxidants, redox reactions, and methods for their quantification. *Toxicologic Pathology* **30(6):**620–650 DOI 10.1080/01926230290166724.

Kubelka M, Motlík J, Schultz RM, Pavlok A. 2000. Butyrolactone I reversibly inhibits meiotic maturation of bovine oocytes without influencing chromosome condensation activity. *Biology of Reproduction* **62(2):**292–302 DOI 10.1095/biolreprod62.2.292.

Li L, Hsu A, Moore PK. 2009. Actions and interactions of nitric oxide, carbon monoxide and hydrogen sulphide in the cardiovascular system and in inflammation—a tale of three gases! *Pharmacology & Therapeutics* **123(3):**386–400 DOI 10.1016/j.pharmthera.2009.05.005.

Li XX, Lee K-B, Lee JH, Kim KJ, Kim EY, Han K-W, Park K-S, Yu J, Kim MK. 2014. Glutathione and cysteine enhance porcine preimplantation embryo development *in vitro* after intracytoplasmic sperm injection. *Theriogenology* **81(2):**309–314 DOI 10.1016/j.theriogenology.2013.09.030.

Louis XL, Murphy R, Thandapilly SJ, Yu L, Netticadan T. 2012. Garlic extracts prevent oxidative stress, hypertrophy and apoptosis in cardiomyocytes: a role for nitric oxide and hydrogen sulfide. *BMC Complementary and Alternative Medicine* **12(1):**140 DOI 10.1186/1472-6882-12-140.

Markides CSA, Roy D, Liehr JG. 1998. Concentration dependence of prooxidant and antioxidant properties of catecholestrogens. *Archives of Biochemistry and Biophysics* **360(1):**105–112 DOI 10.1006/abbi.1998.0934.

Mizuguchi S, Takemura S, Minamiyama Y, Kodai S, Tsukioka T, Inoue K, Okada S, Suehiro S. 2006. S-allyl cysteine attenuated CCl_4-induced oxidative stress and pulmonary fibrosis in rats. *Biofactors* **26(1):**81–92 DOI 10.1002/biof.5520260108.

Morado SA, Cetica PD, Beconi MT, Dalvit GC. 2009. Reactive oxygen species in bovine oocyte maturation *in vitro*. *Reproduction, Fertility and Development* **21(4):**608–614 DOI 10.1071/RD08198.

Nevoral J, Petr J, Gelaude A, Bodart J-F, Kucerova-Chrpova V, Sedmikova M, Krejcova T, Kolbabova T, Dvorakova M, Vyskocilova A, Weingartova I, Krivohlavkova L,

Zalmanova T, Jilek F. 2014. Dual effects of hydrogen sulfide donor on meiosis and cumulus expansion of porcine cumulus-oocyte complexes. *PLoS ONE* **9(7):**e99613 DOI 10.1371/journal.pone.0099613.

Pasqualotto EB, Agarwal A, Sharma RK, Izzo VM, Pinotti JA, Joshi NJ, Rose BI. 2004. Effect of oxidative stress in follicular fluid on the outcome of assisted reproductive procedures. *Fertility and Sterility* **81(4):**973–976 DOI 10.1016/j.fertnstert.2003.11.021.

Paul BD, Snyder SH. 2012. H_2S signalling through protein sulfhydration and beyond. *Nature Reviews Molecular Cell Biology* **13(8):**499–507 DOI 10.1038/nrm3391.

Szabó C. 2007. Hydrogen sulphide and its therapeutic potential. *Nature Reviews Drug Discovery* **6(11):**917–935 DOI 10.1038/nrd2425.

Takami M, Preston SL, Toyloy VA, Behrman HR. 1999. Antioxidants reversibly inhibit the spontaneous resumption of meiosis. *American Journal of Physiology* **276(4):**E684–E688.

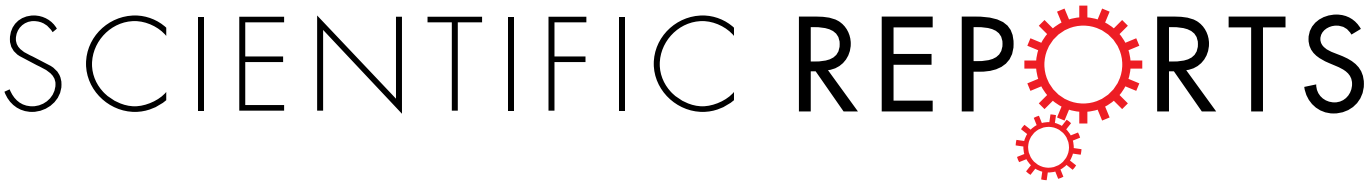
Takemura S, Ichikawa H, Naito Y, Takagi T, Yoshikawa T, Minamiyama Y. 2014. S-allyl cysteine ameliorates the quality of sperm and provides protection from age-related sperm dysfunction and oxidative stress in rats. *Journal of Clinical Biochemistry and Nutrition* **55(3):**155–161 DOI 10.3164/jcbn.14-39.

Takenaka M, Horiuchi T, Yanagimachi R. 2007. Effects of light on development of mammalian zygotes. *Proceedings of the National Academy of Science of the United States of America* **104(36):**14289–14293 DOI 10.1073/pnas.0706687104.

Tsai SJ, Chiu CP, Yang HT, Yin MC. 2011. *s*-Allyl cysteine, *s*-ethyl cysteine, and *s*-propyl cysteine alleviate β-amyloid, glycative, and oxidative injury in brain of mice treated by D-galactose. *Journal of Agricultural and Food Chemistry* **59(11):**6319–6326 DOI 10.1021/jf201160a.

Wassarman PM. 1988. The mammalian ovum. In: Knobil E, Neil J, eds. *The Physiology of Reproduction*. New York: Raven Press.

Whitaker BD, Casey SJ, Taupier R. 2012. The effects of N-acetyl-L-cysteine supplementation on *in vitro* porcine oocyte maturation and subsequent fertilisation and embryonic development. *Reproduction, Fertility and Development* **24(8):**1048–1054 DOI 10.1071/RD12002.



OPEN

Bisphenol S negatively affects the meotic maturation of pig oocytes

Tereza Žalmanová[1], Kristýna Hošková[1], Jan Nevoral[1,2], Kateřina Adámková[1], Tomáš Kott[3], Miloslav Šulc[4], Zora Kotíková[4], Šárka Prokešová[1], František Jílek[1], Milena Králíčková[2] & Jaroslav Petr[3]

Received: 28 October 2016
Accepted: 1 March 2017
Published online: 28 March 2017

Bisphenol A (BPA), a chemical component of plastics, is a widely distributed environmental pollutant and contaminant of water, air, and food that negatively impacts human health. Concerns regarding BPA have led to the use of BPA-free alternatives, one of which is bisphenol S (BPS). However, the effects of BPS are not well characterized, and its specific effects on reproduction and fertility remain unknown. It is therefore necessary to evaluate any effects of BPS on mammalian oocytes. The present study is the first to demonstrate the markedly negative effects of BPS on pig oocyte maturation *in vitro*, even at doses lower than those humans are exposed to in the environment. Our results demonstrate (1) an effect of BPS on the course of the meiotic cell cycle; (2) the failure of tubulin fibre formation, which controls proper chromosome movement; (3) changes in the supply of maternal mRNA; (4) changes in the protein amounts and distribution of oestrogen receptors α and β and of aromatase; and (5) disrupted cumulus cell expansion. Thus, these results confirm that BPS is an example of regrettable substitution because this substance exerts similar or even worse negative effects than those of the material it replaced.

Many anthropogenic substances introduced to the environment exert endocrine-disrupting effects and negatively affect animal and human health by altering the functions of various endogenous hormones, even at very low doses[1]. Because reproduction is subject to complex endocrine regulation, the effects of low-dose endocrine disruptors may severely impact reproductive processes. Bisphenol A (BPA) is a known endocrine disruptor and a component of most plastics, allowing it to reach not only the domestic environment but also water and food supplies[2]. In addition to affecting many other physiological processes[3], BPA may significantly affect reproductive physiology[4–6]. Low-dose exposure to BPA during prenatal and neonatal development has been linked to a wide variety of effects, including alterations in brain sexual differentiation, male and female reproductive tract defects, pregnancy complications, and meiotic abnormalities in foetal oocytes[7,8]. Oestrogenic properties of BPA are known as one of known molecular action in reproductive system[9]. For these reasons, the use of BPA was restricted, and a number of products are sold with the guarantee that they are BPA-free.

In BPA-free products, the forbidden BPA has been replaced by other substances, of which the most widely used is bisphenol S (BPS)[10]. BPS is used compound in common plastics, canned items, receipt papers and many others[8]. Therefore, global production of BPS is rising sharply[11]. Massive exposure to BPS has been observed in many populations worldwide[12]. BPS simulates the actions of oestrogens, and a number of studies have demonstrated the negative effects of BPS on a wide range of physiological processes[13]. There are many indications that BPS has become a "regrettable substitution", specifically, that the endocrine disruptor BPA has been replaced by a substance that exerts vigorous endocrine-disruptive effects[14,15]. A recent examination of urine samples in the United States and Asia confirmed previous work showing that 93% of people had detectable levels of BPA but surprisingly showed that 81% had detectable levels of BPS[16]. Moreover, BPS has been detected in human blood serum[17]. Thus, its possible effects on highly sensitive physiological functions, such as reproduction, must be elucidated. Meiotic maturation of oocytes is a highly sensitive reproductive physiological process. The presence of BPS in body fluids prompts the question of whether BPS exposure disrupts oocyte maturation. Given this, it is troubling that information regarding the influence of BPS on mammalian oocytes remains lacking.

[1]Czech University of Life Sciences Prague, Faculty of Agrobiology, Food and Natural Resources, Department of Veterinary Sciences, Prague, Czech Republic. [2]Charles University in Prague, Faculty of Medicine in Pilsen, Biomedical Center and Department of Histology and Embryology, Pilsen, Czech Republic. [3]Institute of Animal Science, Prague, Czech Republic. [4]Czech University of Life Sciences Prague, Faculty of Agrobiology, Food and Natural Resources, Department of Chemistry, Prague, Czech Republic. Tereza Žalmanová and Kristýna Hošková contributed equally to this work. Correspondence and requests for materials should be addressed to T.Ž. (email: zalmanovat@af.czu.cz)

The process by which mammalian oocytes form a complex with surrounding cumulus cells to prepare for fertilization is dependent on hormonal stimuli. Utilizing stored RNA and stored and newly synthesized proteins, oocytes undergo a complex series of processes termed meiotic maturation[18], which includes breakdown of the nuclear membrane and chromatin condensation (germinal vesicle (GV) breakdown), as well as the formation of microtubule-organizing centres for spindle division. Chromosomal movement is necessary for meiosis I and meiosis II and for extrusion of the polar body[19]. Flawless tubulin function during these processes is required for the meiotic cell cycle to proceed successfully[20]. Oestrogens and aromatase regulate the maturation of mammalian oocytes, which plays a crucial role in steroidogenesis[21]. Therefore, the influence of BPS on oestrogen receptors and aromatase demands attention. Cumulus cells also respond to disrupted hormonal signalling by altering the production of hyaluronic acid (HA), the most abundant compound of their extracellular matrix[22].

We selected pig oocytes as a suitable model to study endocrine disruption in mammalian oocytes. The maturation time of a pig oocyte is longer than that of the commonly used mouse model, therefore providing an opportunity to carry out a more detailed study of the cell cycle. Pig oocytes are also larger in size, permitting the more detailed evaluation of phenomena related to tubulin alterations resulting from greater functional distances between the meiotic spindle and chromosomes, which the microtubules must span. In addition, pig oocytes are physiologically more similar to human oocytes than mouse oocytes, and thus our results provide a more solid basis for human reproductive research[23].

The aim of this study was to explore the effects of BPS on the *in vitro* maturation of porcine oocytes. The results reported here are the first to demonstrate the detrimental effects of BPS on the maturation of mammalian oocytes *in vitro*, indicating the regrettable substitution of BPS for BPA merits our attention with respect to mammalian reproduction.

Results

Our analysis of pig follicular fluid confirmed the absence of BPS. On the basis of this observation (see Supplementary Tables S1 and S2), we suspected that cumulus-oocyte complexes were not influenced by BPS before isolation. Moreover, the viability of oocyte and cumulus cells was tested. After 24 and 48 h of *in vitro* culture, none of the BPS treatments (3 nM, 300 nM or 30 μM) influenced the viability of oocytes and cumulus cells (see Supplementary Table S3A and S3B).

Both progression to MI and MII were sensitive to BPS. Under *in vitro* conditions, pig oocytes mature to metaphase I (MI) after 24 h and to metaphase II (MII) after 48 h. Cumulus–oocyte complexes (COCs) treated with various concentrations of BPS (3 nM, 300 nM or 30 μM) exhibited a significant dose-dependent decrease in MI and MII stage achievement after 24 and 48 h of *in vitro* culture. BPS-treated oocytes (300 nM and 30 μM) did not resume meiosis after 24 h of *in vitro* culture. However, after 48 h of *in vitro* culture, all BPS-treated oocytes initiated meiotic maturation and matured to at least MI (Fig. 1A,B).

After 72 h of culture (Fig. 1C), maturation was not only delayed but also disrupted and blocked by BPS in all used concentrations. This meiotic block was irreversible because maturation did not improve even after 48 h of culture with BPS followed by culture in a BPS-free medium (see Supplementary Fig. S1A). Both progression to MI and to MII are sensitive to all tested concentration of BPS: maturation decreased in COCs exposed to BPS for the first 24 h (Fig. S1B) or during the second 24 h of 48 h of overall culture in dose dependent manner (Fig. S1C).

α-tubulin assembly during porcine oocyte maturation after BPS treatment. Faultless organization of tubulin filaments and chromosomes in the spindle apparatus is required for correct meiotic maturation to be achieved. We observed several types of defects, including swollen chromosomes and irregular organization, decreased numbers of tubulin filaments, spindles in a circular formation or astral arrangement, elongated metaphase plates, and reduced spindle size. These phenomena were apparent in both MI (Fig. 2A) and MII (Fig. 2B) oocytes and were present even in the 3 nM BPS treatment group after 24 and 48 h of *in vitro* culture, respectively. BPS dramatically affects the formation and structure of the meiotic spindle (see Supplementary Video 1).

Effects of BPS on the amount of mRNA for oestrogen receptors and aromatase. The oocyte is transcriptionally inactive during meiotic maturation; therefore, correct meiotic maturation is completely dependent on maternal reserves of gene transcripts. Important targets of the oestrogenic effects of BPS are the mRNA transcripts for ERα, ERβ, and aromatase. Our results indicated the presence of mRNA transcripts for ERα, ERβ, and aromatase in oocytes and cumulus cells, whose responses to BPS treatment differed based on transcript amounts. Notably, the amount of ERα transcripts in oocytes was dramatically decreased after BPS treatment regardless of concentration. Moreover, the amount of aromatase transcripts was dramatically decreased in oocytes treated with BPS concentrations of 3 nM or 300 nM. No changes in the amount of ERβ transcripts were observed in oocytes. In the cumulus cells surrounding the oocytes, mRNAs of aromatase and ERβ decreased after 30 μM BPS treatment (Fig. 3A–C).

Effects of BPS on the expression and redistribution of ERα, ERβ, and aromatase during the maturation of porcine oocytes. The presence of ERα, ERβ, and aromatase was observed throughout the entire meiotic maturation process. The expression and distribution of ERα and ERβ were significantly altered during *in vitro* culture. Notably, these changes were detected during the first meiotic division in which treatment with 30 μM BPS significantly increased the signal intensity of ERα and ERβ. Moreover, the 300 nM BPS treatment also affected these two factors in MI and MII oocytes. Differences were also observed in aromatase expression and distribution within MI oocytes treated with 3 nM BPS (Fig. 4A–F).

Changes in HA-derived cumulus expansion after BPS treatment. During oocyte maturation *in vitro*, cumulus cells produce large amounts of extracellular matrix in which HA is the most abundant compound.

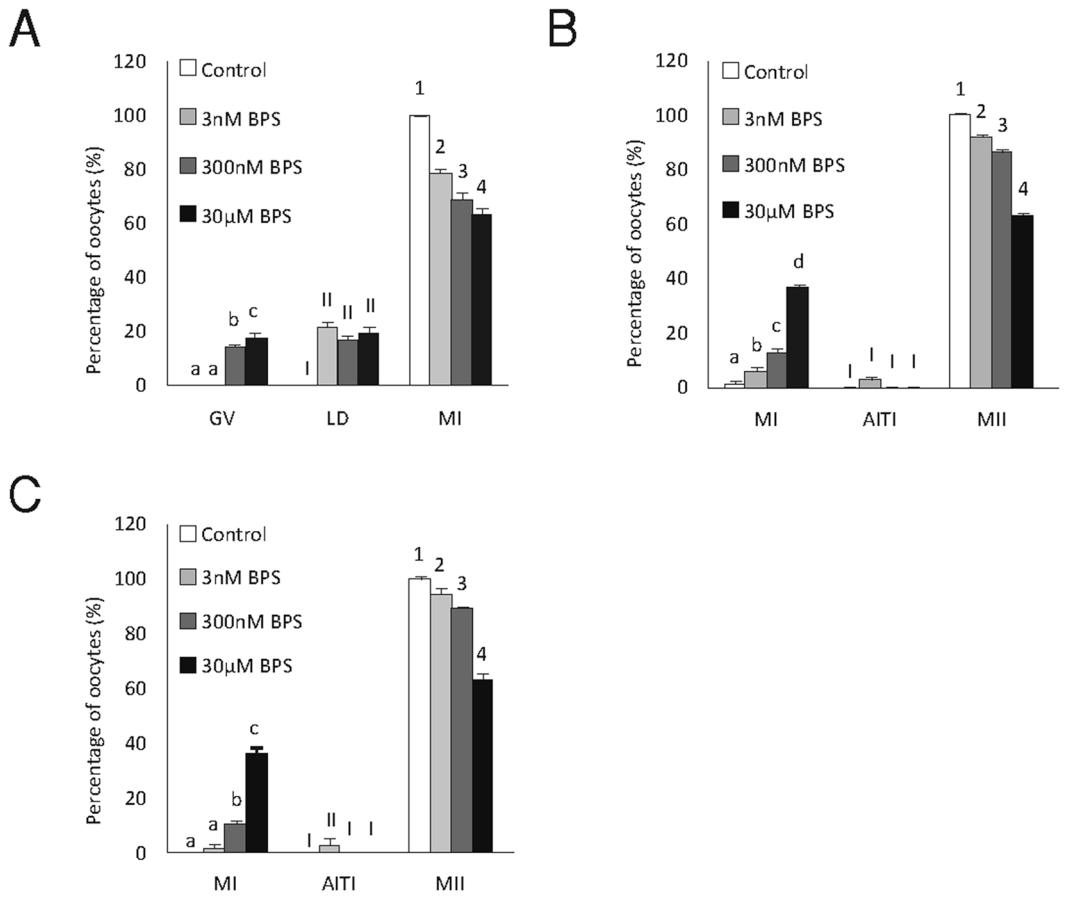


Figure 1. Effects of BPS on the meiotic maturation of oocytes. Effects of BPS (3 nM, 300 nM, and 30 μM) on the stages of meiotic maturation achieved by oocytes cultured for (**A**) 24 h, (**B**) 48 h, and (**C**) 72 h *in vitro*. GV – germinal vesicle, LD – late diakinesis, MI – metaphase I, AITI – anaphase I–telophase I, MII – metaphase II. The data are expressed as the mean ± SEM from four independent experiments, n = 120 oocytes per group. Different superscripts denote the statistical significance among experimental groups within the same stage of meiotic maturation ($P < 0.05$).

In the presence of BPS, HA production in cumulus cells significantly changed. After 300 nM BPS treatment, HA production increased after 24 and 48 h of *in vitro* culture. Interestingly, the other tested BPS concentrations (3 nM and 30 μM) did not influence HA production in COCs (Fig. 3D).

Discussion

To the best of our knowledge, this is the first study to investigate the relationship between BPS exposure and the maturation of mammalian oocytes. Our results demonstrate the markedly negative impact of BPS on pig oocyte maturation *in vitro*, specifically in terms of cell cycle blockade, cytoskeletal disruption, changes in the mRNA levels of key BPS targets, and changes in cumulus expansion. The negative effects of BPS on pig oocyte maturation were also apparent at concentrations that were orders of magnitude lower than BPS concentrations observed in human blood serum and urine[17]. Pig oocyte physiology shares many similarities with that of human oocytes. Importantly, pig oocytes have high sensitivity to the negative effects of BPA during *in vitro* maturation[24]. Therefore, our results are reliably applicable to human reproduction.

Because oestrogens are highly prevalent in the environment, it was necessary to exclude background BPS in our experiments to evaluate the effects of low doses of BPS. Environmental factors altering the composition of follicular fluid harm oocyte competence, either via direct effects on the oocyte itself or by indirectly affecting follicular cells or hormonal actions. Therefore, knowledge of the history of oocytes placed into an *in vitro* maturation system is required[25]. The follicular fluid creating the microenvironment for our porcine oocytes was analytically demonstrated to be free of BPS. The oocytes used in our experiments were exposed only to the BPS concentrations that were added into the culture medium (see Supplementary Tables S1 and S2). In addition to this fact, BPS doses used in our experiments respect concentrations measured in human blood serum and urine (0.8–84 nM)[15,16].

The effects of BPS on the course of the meiotic cell cycle were evaluated at different time intervals during *in vitro* oocyte maturation. Under *in vitro* conditions, BPS blocked the maturation of some oocytes in MI and/or at the exit from MI. After 24 h of culture, oocytes reached the MI stage with decreased success, whereas all oocytes reached the MI stage after 48 h of maturation, but a portion did not continue in meiosis up to MII. These effects

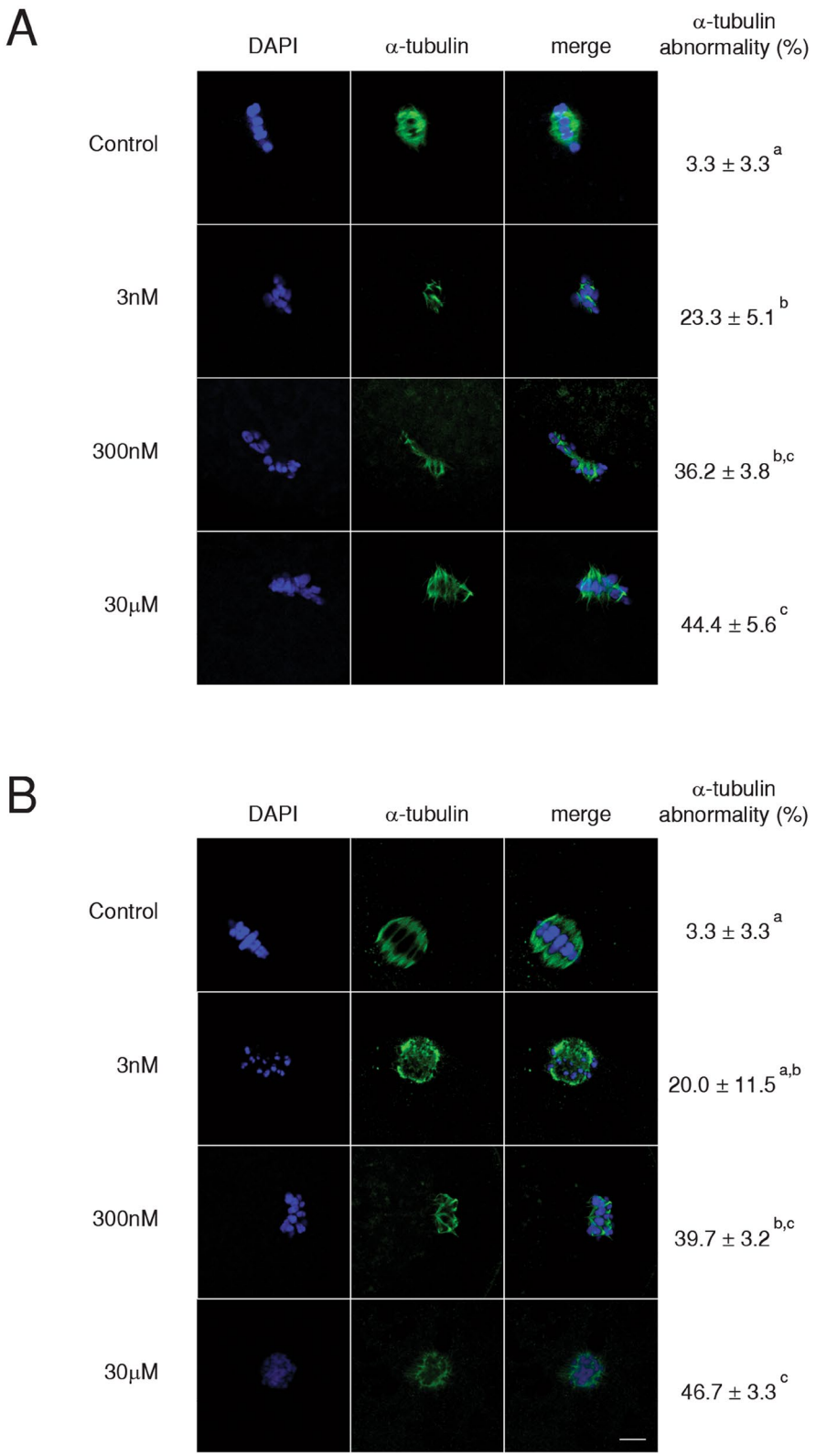


Figure 2. Effects of BPS on meiotic spindle formation during the maturation of porcine oocytes. Representative pictures showing defects in the morphology of spindle organization and chromosome alignment in oocytes after 24 h (**A**) or 48 h (**B**) of culture *in vitro* after BPS (3 nM, 300 nM, and 30 μM) treatment. Green colour indicates α-tubulin, blue indicates DAPI. Scale bar = 10 μm. Percentage of α-tubulin abnormalities after 24 h (n = 83) and 48 h (n = 82) of culture *in vitro* are presented to the right side of the images. The data are expressed as the mean ± SEM of three independent experiments. Different superscript letters denote statistical significance ($P < 0.05$).

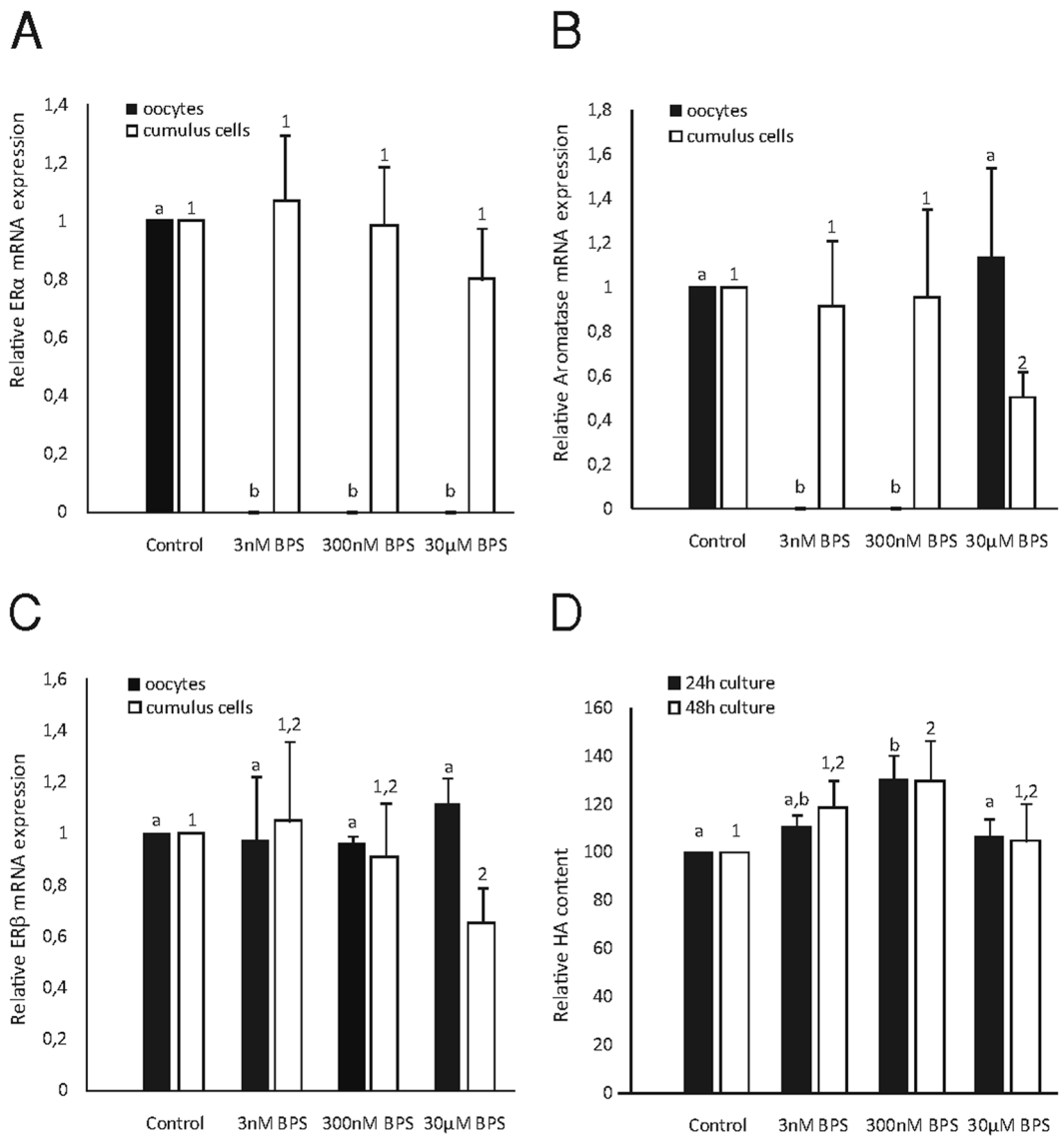


Figure 3. Effects of BPS on mRNA expression levels of selected genes and cumulus cell expansion. Effects of BPS (3 nM, 300 nM, and 30 μM) on the relative mRNA expression of (**A**) ERα, (**B**) ERβ, and (**C**) aromatase in oocytes and cumulus cells cultured for 48 h *in vitro*. The data are expressed from three independent experiments, with a total of n = 150 oocytes per group. Different letters and numbers denote the statistical significance among experimental groups for oocytes and cumulus cells, respectively ($P < 0.05$). (**D**) Effects of BPS on HA content in COCs after 24 and 48 h of *in vitro* culture. Different letters and numbers denote the statistical significance among experimental groups for 24 and 48 h of *in vitro* culture, respectively ($P < 0.05$).

were dose-dependent. Given the observed effects of BPS on pig oocyte maturation, the period around MI appears to be critical. This phenomenon was also demonstrated by our experiments in which BPS exerted substantial effects on *in vitro* oocyte maturation during the first 24 h period and the second 24 h period (see Supplementary Fig. S1). However, it was only possible to determine whether these effects were attributable to a slowing of the cell cycle or meiotic maturation blockade after culture was prolonged beyond 48 h. Significantly, a proportion of oocytes exposed to BPS remained in the MI or anaphase I/telophase I stages. Therefore, BPS not only causes a slowing of pig oocyte maturation *in vitro*, similar to that observed during the maturation of mouse oocytes in the presence of BPA[26] or bisphenol AF[27], but BPS also permanently blocks the course of maturation in a significant portion of oocytes. Similar effects have been observed for the maturation of pig oocytes in BPA presence[24]. Although the effects of low doses of endocrine disruptors are not surprising[28], we demonstrated significantly negative effects with a very low dose of BPS (3 nM), which has not previously been observed in experiments investigating the effects of BPA on mammalian oocyte maturation.

Oocyte sensitivity to BPS during the period surrounding MI appears to be related to meiotic spindle formation. In our experiments, even oocytes cultivated in only 3 nM BPS were distinctly damaged. Based on our results, BPS impairs meiotic spindle creation in pig oocytes and causes irregularities in the arrangement of tubulin fibres. Similar effects on chromosome congression failure *in vivo*[29] and *in vitro*[26,30] were observed during mouse oocyte

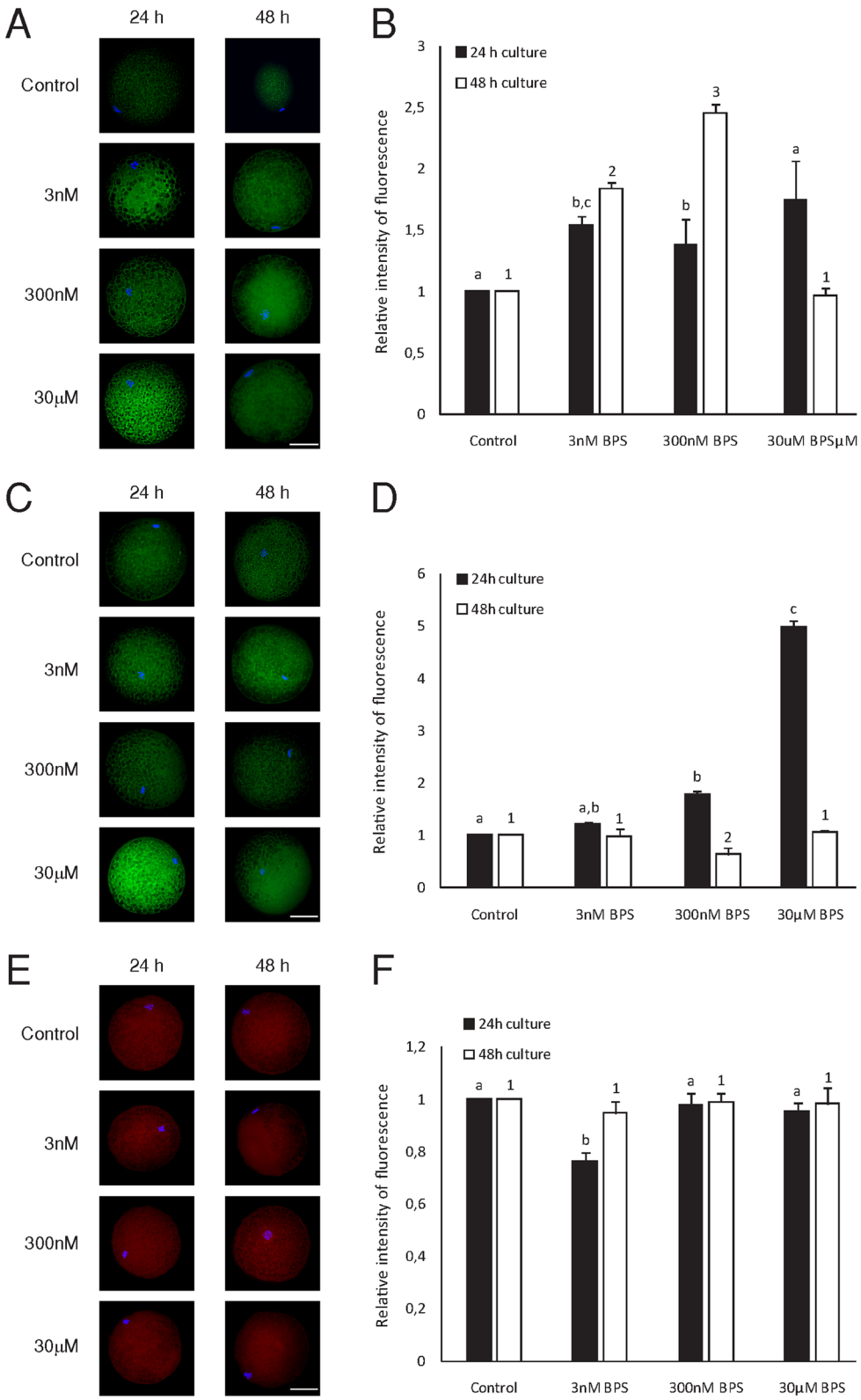


Figure 4. Effects of BPS on ERα, ERα, and aromatase during oocyte maturation. Representative pictures showing changes in the distribution of ERα (**A**), ERβ (**C**), and aromatase (**E**) in oocytes cultured for 24 h and 48 h *in vitro* after BPS (3 nM, 300 nM, and 30 μM) treatment. Green colour indicates ERα and ERβ, red indicates aromatase, and blue indicates chromatin. Scale bar = 50 μM. Graphs (**B**), (**D**), and (**F**) represent differences in the relative fluorescence intensities of ERα, ERβ, and aromatase. The data are expressed as the mean ± SEM of three independent experiments in which at least 20 oocytes were analysed. Different superscript letters denote the statistical significance among experimental groups for 24 and 48 h of *in vitro* culture, respectively ($P < 0.05$).

maturation in the presence of BPA; meiotic spindle abnormalities apparently resulted from spindle checkpoint control failure. During the *in vitro* maturation of pig oocytes, BPA exerted negative effects on cell cycle progression, spindle architecture, and chromosome organization[24]. These effects may be attributable to the influence of BPS on oestrogens: oestrogens, specifically oestradiol, affect the regulation of mammalian oocyte maturation *in vitro*[31,32], and increased concentrations result in meiotic spindle defects[32]. In our experiments, individual meiotic spindle defect frequencies were not linearly dependent on dosage, as is often the case with endocrine disruptors[33–35]. A similar non-linear effect was observed in terms of the effects of BPA on human oocyte maturation *in vitro*[36]. In our study, we demonstrated for the first time the impact of BPS on cytoskeletal structures and noted equally dangerous effects compared to those confirmed in studies investigating BPA.

Our results demonstrate the presence of mRNA transcripts for frequent targets of endocrine disruptors, specifically ERα, ERβ, and aromatase, both in oocytes and in cumulus cells. After *in vitro* culture with BPS at low concentrations, mRNA transcripts for ERα and aromatase were no longer detectable in oocytes. This phenomenon was related to the non-linear effects induced by the endocrinologically disruptive actions of BPS. The ability of BPS to regulate mRNA expression was previously confirmed only in somatic cells[37]. BPA alters the global supply of gene transcripts connected to key cell processes in oocytes[38]. Altered signalling during processes leading to destabilization of the overall maternal stock of mRNA in oocytes[39] or its selective degradation[40] may be responsible for the decrease in mRNA transcript levels that we observed. This phenomenon might also be explained by high levels of translation and the required presence of proteins to sustain or release from the first meiotic block[41]. Somatic cumulus cells play a role in transferring transcripts into the oocyte and also enlarge maternal mRNA stocks within the oocyte[42]. Our results also demonstrate the decreased expression of mRNA transcripts for ERβ and aromatase in cumulus cells surrounding oocytes exposed to 30 μM BPS, which may result in the decreased transport of these mRNAs from the cumulus cells into the oocyte. These effects are potentially attributable to toxicity, which would be in accordance with the effects of BPA described in somatic cell lines[43]. The different effects of BPS on ERα and ERβ transcripts may be related to the affinity of ERs to BPS[44]. Thus, BPS may trigger diverse translation responses. The decrease in ERα transcripts may be explained by a nonlinear relationship between the number of bound receptors and the strongest observable biological effect[35]. Similarly, aromatase transcript expression has also shown a non-monotonic effect, in which low doses appear to be more effective than high doses in altering transcript levels. Decreases in the amount of transcripts can be explained by increased translation as well as disruption of transcript stability (*e.g.*, due to polyadenylation of mRNAs)[18,45]. BPS may thus affect both of these mechanisms of transcription regulation. In general, the same concentrations of BPS may exert even more damaging impacts on oocytes than on cumulus cells in terms of decreasing mRNA transcript levels, suggesting female gametes are more sensitive than somatic cells to the endocrinologically disruptive actions of BPS. At the same time, there may be different BPS signalling mechanisms in somatic cells versus oocytes.

Our results indicate direct ERα and ERβ protein expression in oocytes. Moreover, BPS also possesses the ability to influence meiotic maturation by targeting oestrogen receptors. Culture with BPS disrupts the expression of ERα and ERβ, as seen in MI and MII. In somatic cells, BPS acts as a weak agonist of oestrogen receptors[46] present in a number of tissue types. During somatic cell mitosis, ERα regulates chromosome alignment and spindle dynamics by stabilizing microtubules during metaphase[47]. The absence of ERα mRNA in mature oocytes may be associated with increased ERα signal intensity during *in vitro* maturation, thus suggesting transcript depletion during ERα translation. Moreover, BPS-induced alterations in ERα signal intensity after 24 and 48 hr of *in vitro* culture may affect microtubule function, thus causing the spindle malformations[47] observed in our experiments. Both oestrogen receptors clearly increased after 24 hr of *in vitro* culture with BPS. Although the amount of ERα protein was accompanied by decreases in mRNA, ERβ mRNA was not affected. This observation suggests that the ubiquitin-proteasome system[48] may be targeted when proteolytic degradation of ERβ is protracted. However, the amount of ERβ protein did not increase after 48 hr of *in vitro* culture; in contrast, stimulation of proteolytic degradation appeared to occur after 300 nM BPS treatment, in a manner potentially promoted by receptor saturation[49,50]. Noticeably, BPS, simulating oestrogen action, affect dynamics of oestrogen receptors due to both post-transcriptional and post-translational regulation[50,51].

In addition to endocrine disruptors, the expression of aromatase, which is responsible for steroidogenesis, is affected in porcine oocytes. Therefore, cross-talk between aromatase-derived oestrogens and endocrine disruptors is a target of BPS in porcine oocytes, and our evidence points to endocrinological disruption by BPS, which affects mammalian oocyte maturation *in vitro*. According to our results, BPS increases the levels of retained HA in cumulus–oocyte complexes. This effect was observed after 24 h and 48 h of *in vitro* culture at concentration of 300 nM BPS. BPA also alters HA levels by decreasing the amounts of retained HA[22] and suppressing the cumulus expansion of pig COCs[24]. Presumably, 300 nM BPS may mimic hormonal stimulation of cumulus expansion within 24 h cultured oocytes. Futhermore, BPS can also affect paracrine regulation factors (*i.e.*, insulin-like growth factor and growth differentiation factor-9)[52,53] and other key molecules, such as hyaluronan synthase-2, cAMP, and/or microRNAs[54,55]. The proposed BPS sensitivity of cumulus expansion regulatory mechanisms is consistent with earlier observations on the effects of other endocrine disruptors on cumulus-oocyte complexes and cumulus expansion[22]. BPS exerts different effects as oocyte maturation progresses. The non-linear effect of BPS is apparent when 3 nM and 30 μM BPS treatments do not show significant effects. This differing mechanism may also underlie the effects on mRNA expression observed at the low doses evaluated in our study. Furthermore, our findings correspond to the aforementioned non-linear effects of BPS.

In conclusion, based on the results of our study, mammalian oocytes are highly sensitive to the effects of BPS. This is the first study describing the impact of low doses of BPS on mammals. The presented results help to clarify the mechanism by which endocrine disruptors influence mammalian reproduction and suggest that the ever-increasing use of BPS does not constitute a safer alternative to BPA.

Methods

Reagents. Chemicals were obtained from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise noted.

Porcine oocyte isolation and culture. The authors declare that the present study was carried out in accordance with the current laws of the Czech Republic and all the experimental protocols were approved by the Ethics Committee at the Czech University of Life Sciences Prague. Porcine ovaries were collected from pre-pubertal gilts at a local slaughterhouse. COCs were aspirated from medium-sized follicles using a 20-gauge needle. Only oocytes surrounded by several layers of cumulus cells and uniform ooplasm were selected for further study. Oocytes were cultured in M199 medium supplemented with sodium bicarbonate (0.039 mL of a 7.0% solution per 1 mL of medium), calcium lactate (0.6 mg/mL), gentamicin (0.025 mg/mL), HEPES (1.5 mg/mL), 13.5 IU of eCG plus 6.6 IU of hCG/mL (P.G. 600 Intervet, Boxmeer, Netherlands), and 5% foetal calf serum. Based on our preliminary experiments (data not shown), COCs were treated with BPS in following concentrations: 30 pM, 3 nM, 300 nM, and 30 μM, dissolved in DMSO to its final concentration of 0.1%. Vehicle control when COCs cultivated in medium with equal DMSO concentration was used. The oocytes were cultured for 24, 48, or 72 h in 5.0% CO_2 at 39 °C.

Oocyte evaluation. After culture, the oocytes were denuded from surrounding cumulus cells by pipetting. Thereafter, oocytes were mounted onto slides, fixed with acetic alcohol (1:3, v/v) for at least 24 h, and stained with 1.0% orcein. The oocytes were examined under a phase-contrast microscope (magnification 400x). The stages of oocyte nuclear maturation, specifically GV, late diakinesis (LD), MI, anaphase I (AI), telophase I (TI), and MII, were evaluated in accordance with previously described criteria[56].

Trypan blue staining of oocytes and cumulus cells. After 24 or 48 hr of *in vitro* cultivation, COCs (15 per group) were incubated with a 0.2 (w/v) solution of Trypan blue for 10 min. After incubation, oocytes were denuded, and Trypan blue-positive cells were counted. Cumulus cells were washed three times in PBS, and Trypan blue-positive cells were counted with a Thoma chamber when one hundred cells were evaluated.

Oocyte immunofluorescence and imaging. After culture, oocytes were treated with 0.5% pronase to remove the *zona pellucida* and further processed as previously described[57] with slight modifications. Oocytes were permeabilised and blocked (in 0.1% Triton X-100 dissolved in PBS supplemented with 1% and 5% normal goat serum, respectively), and then incubated overnight with the following antibodies (1:200; at 4 °C): anti-α-tubulin (T6199, Sigma-Aldrich), anti-CYP19/aromatase (LS-C188219, LifeSpan BioSciences, Seattle, WA, USA), anti-oestrogen receptor α (ab3575, Abcam, Cambridge, UK), and anti-oestrogen receptor β (ab3576, Abcam). Subsequently, oocytes were washed twice before incubation with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG, FITC-conjugated goat anti-rabbit IgG, and tetramethylrhodamine isothiocyanate (TRITC)-conjugated goat anti-mouse IgG (Thermo Fisher Scientific, Waltham, MA, USA; 1:200). Thereafter, oocytes were washed twice and mounted in Vectashield containing 4′6-diamidino-2-phenylindole (DAPI; Thermo Fisher Scientific). Images were acquired using a Ti-U microscope (Nikon Co., Tokyo, Japan) to detect ERα/ERβ and aromatase. A confocal scanning microscope (Leica, SPE, Germany) was used for α-tubulin visualization. Exposition conditions were the same for each individual protein, and its negative control, which lacked a specific antibody, was processed under comparable conditions. Image analysis was performed using NIS Elements (Laboratory Imaging Ltd, Prague, Czech Republic). Aromatase, ERα, and ERβ signal intensities were normalized to the basal signal intensity of the negative control and compared to those in untreated oocytes.

Western blot analysis. Samples were prepared in accordance with previous study[58]. Briefly, denuded GV oocytes (200 per sample) were placed into 15 μL of sample buffer. Surrounding cumulus cells were processed separately. Samples were heated at 100 °C for 5 min and proteins were separated using 12.5% SDS-PAGE and then electrophoretically transferred to a nitrocellulose membrane (GE Healthcare, Amersham UK). A pre-stained molecular weight standard (Bio-Rad Laboratories, Waltford, UK) was used to verify the molecular weights of the detected proteins. After overnight blocking in 2% milk in phosphate-buffered saline (PBS) containing 0.1% Tween 20, the membrane was incubated for 2 h with primary antibodies at a concentration of 1:500 for β-Actin (#4970, Cell Signaling Technology, Davers, MA, USA; as an internal loading standard), ERα and ERβ, and 1:250 for aromatase. The membrane was incubated with a secondary mouse or rabbit IgG antibody (GE Healthcare) at a concentration of 1:10,000 or 1:40,000, respectively. The proteins were visualized using an ECL Select Western Blotting Detection Kit (GE Healthcare) and a C-Digit Blot Scanner (LI-COR Biosciences, Lincoln, NE, USA).

mRNA analysis. Samples for the quantitative real-time polymerase chain reaction (RT-qPCR) analysis of aromatase, ERα, and ERβ mRNAs were prepared from immature GV and mature MII oocytes (50 oocytes in each group). Concurrently, cumulus cells were employed for the same analysis. RNA was isolated using a 6100 Nucleic Acid PrepStation (Fisher Scientific, USA) in accordance with the instruction manual. Total mRNA was transcribed to cDNA using a High-Capacity cDNA Achieve Kit (Thermo Fisher Scientific) in accordance with the manufacturer's instructions. cDNA was synthesized in a final volume of 100 μL. Sets of specific primers were synthesized in accordance with known sequences to amplify specific products for GAPDH, aromatase, ERα, and ERβ (see Supplementary Table S4). Each PCR reaction was performed in triplicate in a total volume of 10 μL with the gene-specific primers at 500 nM and the TaqMan MGB probe at 200 nM, 5 μL of 2x concentrated Fast TaqMan Universal Master Mix (Thermo Fisher Scientific), 1 μL of cDNA, and nuclease-free water up to a volume of 1 mL. The 7500 Fast Real-Time PCR System (Thermo Fisher Scientific) was utilized for RT-qPCR reactions with the following programme: 95 °C for 20 s followed by 40 cycles of 95 °C for 2 s and 60 °C for 20 s. mRNA expression was quantified for each enzyme using SDS software and the arithmetic formula $2^{-\Delta\Delta CT}$ in accordance with the

comparative Ct method[59] to determine the amount of the target normalized to the GAPDH endogenous control as a reference[45], relative to fully grown GV oocytes and their cumulus cells.

HA measurement and the evaluation of cumulus expansion. Groups of 25 COCs were cultured for 24 or 48 h as described above and processed as previously described[58,60]. Briefly, the COCs were three-times washed in PBS-PVA, and oocytes were mechanically denuded by repeated pipetting. Isolated HA was enzymatically digested with lyase from *Streptomyces hyalurolyticus* (2 IU/mL) at 39 °C overnight. Subsequently, HA solutions were spectrophotometrically measured using a Helios Gamma spectrophotometer (Thermo Fisher Scientific) at 216 nm against a blank consisting of PBS-PVA containing lyase. The quadratic calibration curve was based on five HA standards (0.006–0.1% sodium hyaluronate) digested via the protocol used for the samples. The concentration of HA was expressed as the retained HA relative to the untreated control group.

LC-MS/MS analysis of BPS in porcine follicular fluid. Follicular fluid samples were prepared during oocyte aspiration in accordance with the previously described oocyte collection. The follicular fluid was obtained from three independent aspirating sessions. Subsequently, a sample preparation method described by[61] was employed with modifications. Briefly, samples were centrifuged, and 2 mL of supernatant was added to 1 mL of 200 mM sodium acetate buffer (pH 5.4) together with a ($^{13}C_{12}$) internal standard (10 µL of a 50 ng/mL solution), followed by incubation with 20 µL of beta-glucuronidase/arylsulfatase from *Helix pomatia* (Roche, Mannheim, Germany) for 5 h at 37 °C. Samples were extracted with 2 mL of acetonitrile and 3 mL of ethyl acetate. After sonication (40 kHz for 10 min.) and centrifugation, 4 mL of supernatant was evaporated under nitrogen at 60 °C, and the residue was reconstituted with 0.5 mL of 50% methanol in water. Samples were analysed on a Dionex Ultimate 3000 UHPLC system (Thermo Fisher Scientific) coupled to a 3200 QTRAP triple quadrupole mass spectrometer (AB Sciex, DC). The following liquid chromatography conditions were used: Phenomenex Kinetex C18 column (30 × 2.1 mm, 1.7 µm), column temperature 35 °C, autosampler temperature 10 °C, flow 0.3 mL/min, injection volume 10 µL. Mobile phase (A) was methanol, and phase (B) was water. The following gradient was employed: 0 min 90% B, 0.2 min 90% B, 4.5 min 10% B, 5.5 min 10% B, 6.5 min 90% B, and 8 min 90% B. The following mass spectrometry parameters were used: the ESI source was operated in negative mode at 600 °C, ion spray voltage −3500 V, curtain gas 20 a.u., nebulizer gas 35 a.u., turbo gas 25 a.u., collision gas "medium", ion dwell time 70 ms, ions registered: 249.1/107.9/155.9/92.0 for bisphenol S and 261.1/114.1/98.1/162.1 for the internal standard. The BPS retention time was approximately 2.55 min. Eight-point linear calibration ($r = 0.9999$) ranged from 0.05 ng/mL to 100 ng/mL.

Statistical analysis. The data are presented as the mean ± SEM of at least three independent experiments. The general linear models (GLM) procedure, following the Shapiro-Wilk test of normality, was employed in SAS package 9.3 (SAS Institute Inc., Cary, NC, USA) to analyse data from all experiments. Significant differences among groups were determined using Sheffé's test. $P < 0.05$ was regarded as statistically significant.

References

1. Colborn, T., vom Saal, F. S. & Soto, A. M. Developmental effects of endocrine-disrupting chemicals in wildlife and humans. *Environ Health Perspect* **101**, 378–384 (1993).
2. Huang, S. & Hang, Y. Simultaneous determination of bisphenol A and tetrabromobisphenol A in plastic products using high performance liquid chromatography-mass spectrometry. *Se Pu* **28**, 863–866 (2010).
3. Moriyama, K. *et al*. Thyroid hormone action is disrupted by bisphenol A as an antagonist. *J Clin Endocrinol Metab* **87**, 5185–5190, doi:10.1210/jc.2002-020209 (2002).
4. Machtinger, R. & Orvieto, R. Bisphenol A, oocyte maturation, implantation, and IVF outcome: review of animal and human data. *Reproductive Biomedicine Online* **29**, 404–410, doi:10.1016/j.rbmo.2014.06.013 (2014).
5. Ziv-Gal, A. & Flaws, J. A. Evidence for bisphenol A-induced female infertility: a review (2007–2016). *Fertility and Sterility* **106**, 827–856, doi:10.1016/j.fertnstert.2016.06.027 (2016).
6. Peretz, J. *et al*. Bisphenol A and Reproductive Health: Update of Experimental and Human Evidence, 2007–2013. *Environmental Health Perspectives* **122**, 775–786, doi:10.1289/ehp.1307728 (2014).
7. Hunt, P. A., Susiarjo, M., Rubio, C. & Hassold, T. J. The bisphenol A experience: a primer for the analysis of environmental effects on mammalian reproduction. *Biol Reprod* **81**, 807–813, doi:10.1095/biolreprod.109.077008 (2009).
8. Žalmanová, T. *et al*. Bisphenol S instead of bisphenol A: a story of reproductive disruption by regretable substitution - a review. *Czech Journal of Animal Sciences* **61**, 433–449, doi:10.17221/81/2015-CJAS (2016).
9. Acconcia, F., Pallottini, V. & Marino, M. Molecular Mechanisms of Action of BPA. *Dose-Response* **13**, doi: 10.1177/1559325815610582 (2015).
10. Liao, C., Liu, F. & Kannan, K. Bisphenol s, a new bisphenol analogue, in paper products and currency bills and its association with bisphenol a residues. *Environ Sci Technol* **46**, 6515–6522, doi:10.1021/es300876n (2012).
11. Liao, C. & Kannan, K. Concentrations and profiles of bisphenol A and other bisphenol analogues in foodstuffs from the United States and their implications for human exposure. *J Agric Food Chem* **61**, 4655–4662, doi:10.1021/jf400445n (2013).
12. Wang, W. *et al*. A comparative assessment of human exposure to tetrabromobisphenol A and eight bisphenols including bisphenol A via indoor dust ingestion in twelve countries. *Environ Int* **83**, 183–191, doi:10.1016/j.envint.2015.06.015 (2015).
13. Michałowicz, J., Mokra, K. & Bąk, A. Bisphenol A and its analogs induce morphological and biochemical alterations in human peripheral blood mononuclear cells (*in vitro* study). *Toxicol In Vitro* **29**, 1464–1472, doi:10.1016/j.tiv.2015.05.012 (2015).
14. Kinch, C. D., Ibhazehiebo, K., Jeong, J. H., Habibi, H. R. & Kurrasch, D. M. Low-dose exposure to bisphenol A and replacement bisphenol S induces precocious hypothalamic neurogenesis in embryonic zebrafish. *Proc Natl Acad Sci USA* **112**, 1475–1480, doi:10.1073/pnas.1417731112 (2015).
15. Zimmerman, J. B. & Anastas, P. T. Toward designing safer chemicals. *Science* **347**, 215, doi:10.1126/science.aaa6736 (2015).
16. Liao, C. Y. *et al*. Bisphenol S in Urine from the United States and Seven Asian Countries: Occurrence and Human Exposures. *Environmental Science & Technology* **46**, 6860–6866, doi:10.1021/es301334j (2012).
17. Thayer, K. A. *et al*. Bisphenol A, Bisphenol S, and 4-Hydroxyphenyl 4-Isoprooxyphenyl sulfone (BPSIP) in Urine and Blood of Cashiers. *Environ Health Perspect* **124**, 437–444, doi:10.1289/ehp.1409427 (2016).
18. Susor, A. *et al*. Temporal and spatial regulation of translation in the mammalian oocyte via the mTOR-eIF4F pathway. *Nat Commun* **6**, 6078, doi:10.1038/ncomms7078 (2015).
19. Wassarman, P. M. Fertilization in mammals. *Sci Am* **259**, 78–84 (1988).

20. Kan, R. *et al*. Regulation of mouse oocyte microtubule and organelle dynamics by PADI6 and the cytoplasmic lattices. *Dev Biol* **350**, 311–322, doi:10.1016/j.ydbio.2010.11.033 (2011).
21. Singh, B., Barbe, G. J. & Armstrong, D. T. Factors influencing resumption of meiotic maturation and cumulus expansion of porcine oocyte-cumulus cell complexes *in vitro*. *Mol Reprod Dev* **36**, 113–119, doi:10.1002/mrd.1080360116 (1993).
22. Mlynarcikova, A., Nagyova, E., Fickova, M. & Scsukova, S. Effects of selected endocrine disruptors on meiotic maturation, cumulus expansion, synthesis of hyaluronan and progesterone by porcine oocyte-cumulus complexes. *Toxicology in vitro* **23**, 371–377, doi:10.1016/j.tiv.2008.12.017 (2009).
23. Zhang, Y. *et al*. Small GTPase RhoA regulates cytoskeleton dynamics during porcine oocyte maturation and early embryo development. *Cell Cycle* **13**, 3390–3403, doi:10.4161/15384101.2014.952967 (2014).
24. Wang, T. *et al*. The toxic effects and possible mechanisms of Bisphenol A on oocyte maturation of porcine *in vitro*. *Oncotarget*, doi: 10.18632/oncotarget.8689 (2016).
25. Krisher, R. L. *In vivo* and *in vitro* environmental effects on mammalian oocyte quality. *Annu Rev Anim Biosci* **1**, 393–417, doi:10.1146/annurev-animal-031412-103647 (2013).
26. Can, A., Semiz, O. & Cinar, O. Bisphenol-A induces cell cycle delay and alters centrosome and spindle microtubular organization in oocytes during meiosis. *Mol Hum Reprod* **11**, 389–396, doi:10.1093/molehr/gah179 (2005).
27. Nakano, K. *et al*. Comparison of the effects of BPA and BPAF on oocyte spindle assembly and polar body release in mice. *Zygote* **24**, 172–180, doi:10.1017/S0967199415000027 (2016).
28. Kitamura, S. *et al*. Comparative study of the endocrine-disrupting activity of bisphenol A and 19 related compounds. *Toxicol Sci* **84**, 249–259, doi:10.1093/toxsci/kfi074 (2005).
29. Hunt, P. A. *et al*. Bisphenol a exposure causes meiotic aneuploidy in the female mouse. *Curr Biol* **13**, 546–553 (2003).
30. Eichenlaub-Ritter, U. *et al*. Exposure of mouse oocytes to bisphenol A causes meiotic arrest but not aneuploidy. *Mutat Res* **651**, 82–92, doi:10.1016/j.mrgentox.2007.10.014 (2008).
31. Beker, A. R., Colenbrander, B. & Bevers, M. M. Effect of 17beta-estradiol on the *in vitro* maturation of bovine oocytes. *Theriogenology* **58**, 1663–1673 (2002).
32. Beker-van Woudenberg, A. R., van Tol, H. T., Roelen, B. A., Colenbrander, B. & Bevers, M. M. Estradiol and its membrane-impermeable conjugate (estradiol-bovine serum albumin) during *in vitro* maturation of bovine oocytes: effects on nuclear and cytoplasmic maturation, cytoskeleton, and embryo quality. *Biol Reprod* **70**, 1465–1474, doi:10.1095/biolreprod.103.025684 (2004).
33. Grasselli, F. *et al*. Bisphenol A disrupts granulosa cell function. *Domest Anim Endocrinol* **39**, 34–39, doi:10.1016/j.domaniend.2010.01.004 (2010).
34. vom Saal, F. S. & Welshons, W. V. Endocrine disruptors: Manmade and natural oestrogens: opposite effects on assisted reproduction. *Nat Rev Endocrinol* **12**, 251–252, doi:10.1038/nrendo.2016.38 (2016).
35. Vandenberg, L. N. *et al*. Hormones and endocrine-disrupting chemicals: low-dose effects and nonmonotonic dose responses. *Endocr Rev* **33**, 378–455, doi:10.1210/er.2011-1050 (2012).
36. Machtinger, R. *et al*. Bisphenol-A and human oocyte maturation *in vitro*. *Hum Reprod* **28**, 2735–2745, doi:10.1093/humrep/det312 (2013).
37. Crump, D., Chiu, S. & Williams, K. L. Bisphenol S alters embryonic viability, development, gallbladder size, and messenger RNA expression in chicken embryos exposed via egg injection. *Environ Toxicol Chem* **35**, 1541–1549, doi:10.1002/etc.3313 (2016).
38. Ferris, J., Mahboubi, K., MacLusky, N., King, W. A. & Favetta, L. A. BPA exposure during *in vitro* oocyte maturation results in dose-dependent alterations to embryo development rates, apoptosis rate, sex ratio and gene expression. *Reprod Toxicol* **59**, 128–138, doi:10.1016/j.reprotox.2015.12.002 (2016).
39. Yu, C. *et al*. BTG4 is a meiotic cell cycle-coupled maternal-zygotic-transition licensing factor in oocytes. *Nat Struct Mol Biol* **23**, 387–394, doi:10.1038/nsmb.3204 (2016).
40. Su, Y. Q. *et al*. Selective degradation of transcripts during meiotic maturation of mouse oocytes. *Dev Biol* **302**, 104–117, doi:10.1016/j.ydbio.2006.09.008 (2007).
41. Sun, Q. Y., Lai, L., Bonk, A., Prather, R. S. & Schatten, H. Cytoplasmic changes in relation to nuclear maturation and early embryo developmental potential of porcine oocytes: effects of gonadotropins, cumulus cells, follicular size, and protein synthesis inhibition. *Mol Reprod Dev* **59**, 192–198, doi:10.1002/mrd.1022 (2001).
42. Macaulay, A. D. *et al*. Cumulus Cell Transcripts Transit to the Bovine Oocyte in Preparation for Maturation. *Biol Reprod* **94**, 16, doi:10.1095/biolreprod.114.127571 (2016).
43. La Rosa, P., Pellegrini, M., Totta, P., Acconcia, F. & Marino, M. Xenoestrogens alter estrogen receptor (ER) α intracellular levels. *PLoS One* **9**, e88961, doi:10.1371/journal.pone.0088961 (2014).
44. Goldinger, D. M. *et al*. Endocrine activity of alternatives to BPA found in thermal paper in Switzerland. *Regulatory Toxicology and Pharmacology* **71**, 453–462, doi:10.1016/j.yrtph.2015.01.002 (2015).
45. Kuijk, E. W. *et al*. Validation of reference genes for quantitative RT-PCR studies in porcine oocytes and preimplantation embryos. *BMC Dev Biol* **7**, 58, doi:10.1186/1471-213X-7-58 (2007).
46. Grignard, E., Lapenna, S. & Bremer, S. Weak estrogenic transcriptional activities of Bisphenol A and Bisphenol S. *Toxicol In Vitro* **26**, 727–731, doi:10.1016/j.tiv.2012.03.013 (2012).
47. Zhang, X., Park, H., Han, S. S., Kim, J. W. & Jang, C. Y. ERα regulates chromosome alignment and spindle dynamics during mitosis. *Biochem Biophys Res Commun* **456**, 919–925, doi:10.1016/j.bbrc.2014.12.062 (2015).
48. Alarid, E. T., Bakopoulos, N. & Solodin, N. Proteasome-mediated proteolysis of estrogen receptor: A novel component in autologous down-regulation. *Molecular Endocrinology* **13**, 1522–1534, doi:10.1210/me.13.9.1522 (1999).
49. Tschugguel, W. *et al*. Differential regulation of proteasome-dependent estrogen receptor alpha and beta turnover in cultured human uterine artery endothelial cells. *Journal of Clinical Endocrinology & Metabolism* **88**, 2281–2287, doi:10.1210/jc.2002-021165 (2003).
50. Cheng, G. J. *et al*. Differential regulation of estrogen receptor (ER)alpha and ER beta in primate mammary gland. *Journal of Clinical Endocrinology & Metabolism* **90**, 435–444, doi:10.1210/jc.2004-0861 (2005).
51. Tateishi, Y. *et al*. Turning off estrogen receptor beta-mediated transcription requires estrogen-dependent receptor proteolysis. *Molecular and Cellular Biology* **26**, 7966–7976, doi:10.1128/mcb.00713-06 (2006).
52. Nagyova, E., Prochazka, R. & Vanderhyden, B. C. Oocytectomy does not influence synthesis of hyaluronic acid by pig cumulus cells: Retention of hyaluronic acid after insulin-like growth factor-I treatment in serum-free medium. *Biology of Reproduction* **61**, 569–574, doi:10.1095/biolreprod61.3.569 (1999).
53. Dragovic, R. A. *et al*. Role of oocyte-secreted growth differentiation factor 9 in the regulation of mouse cumulus expansion. *Endocrinology* **146**, 2798–2806, doi:10.1210/en.2005-0098 (2005).
54. Marchal, R. *et al*. Effect of growth hormone (GH) on *in vitro* nuclear and cytoplasmic clocyte maturation, cumulus expansion, hyaluronan synthases, and connexins 32 and 43 expression, and GH receptor messenger RNA expression in equine and porcine species. *Biology of Reproduction* **69**, 1013–1022, doi:10.1095/biolreprod.103.015602 (2003).
55. Yao, G. D. *et al*. MicroRNA-224 is involved in the regulation of mouse cumulus expansion by targeting Ptx3. *Molecular and Cellular Endocrinology* **382**, 244–253, doi:10.1016/j.mce.2013.10.014 (2014).
56. Motlik, J., Fulka, J. & Fléchon, J. E. Changes in intercellular coupling between pig oocytes and cumulus cells during maturation *in vivo* and *in vitro*. *J Reprod Fertil* **76**, 31–37 (1986).

57. Yi, Y. J., Sutovsky, M., Song, W. H. & Sutovsky, P. Protein deubiquitination during oocyte maturation influences sperm function during fertilisation, antipolyspermy defense and embryo development. *Reprod Fertil Dev* **27**, 1154–1167, doi:10.1071/RD14012 (2015).
58. Nevoral, J. *et al*. Endogenously produced hydrogen sulfide is involved in porcine oocyte maturation *in vitro*. *Nitric Oxide* **51**, 24–35, doi:10.1016/j.niox.2015.09.007 (2015).
59. Livak, K. J. & Schmittgen, T. D. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* **25**, 402–408, doi:10.1006/meth.2001.1262 (2001).
60. Zámostná, K. *et al*. A simple method for assessment of hyaluronic acid production by cumulus-oocyte complexes. *Czech Journal of Animal Sciences* **61**, 251–261, doi:10.17221/51/2015-CJAS (2016).
61. Yang, Y. J., Guan, J., Yin, J., Shao, B. & Li, H. Urinary levels of bisphenol analogues in residents living near a manufacturing plant in south China. *Chemosphere* **112**, 481–486, doi:10.1016/j.chemosphere.2014.05.004 (2014).

Acknowledgements

This work was support by the National Agency of Agriculture Sciences (project No. QJ1510138), Ministry of Agriculture of the Czech Republic (project No. MZeRO0714), MEYS (LM2015062 Czech-BioImaging), Charles University in Prague (PRVOUK P-36) and the National Sustainability Program I (NPU I) Nr. LO1503 provided by the Ministry of Education, Youth and Sports of the Czech Republic. We thank I. Horáček for critically reading the manuscript.

Author Contributions

J.P. and T.Ž. conceived and designed research; J.P., T.Ž., K.H., J.N., K.A., T.K., Š.P. performed the experiments, M.Š., Z.K. analysed follicular fluid, J.P., T.Ž., K.H., J.N., F.J. and M.K. wrote the manuscript. T.Ž. and K.H. authors contributed equally to this work.

Additional Information

Supplementary information accompanies this paper at doi:10.1038/s41598-017-00570-5

Competing Interests: The authors declare that they have no competing interests.

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

Contents lists available at ScienceDirect

Prostaglandins and Other Lipid Mediators

journal homepage: www.elsevier.com

Original Research Article

Prostaglandin E2 stimulates the expression of cumulus expansion-related genes in pigs: the role of protein kinase B

Milan Blaha[a, *], Radek Prochazka[a], Katerina Adamkova[b], Jan Nevoral[b, c, d], Lucie Nemcova[a]

[a] Laboratory of Developmental Biology, Institute of Animal Physiology and Genetics, Academy of Sciences of the Czech Republic, Rumburska 89, 277 21 Libechov, Czech Republic
[b] Department of Veterinary Sciences, Faculty of Agrobiology, Food and Natural Resources, Czech University of Life Sciences in Prague, Kamycka 129, 265 21 Prague 6-Suchdol, Czech Republic
[c] Laboratory of Reproductive Medicine of Biomedical Center, Faculty of Medicine in Pilsen, Charles University, Karlovarska 48, 301 66 Pilsen, Czech Republic
[d] Department of Histology and Embryology, Faculty of Medicine in Pilsen, Charles University, Karlovarska 48, 301 66 Pilsen, Czech Republic

ARTICLE INFO

Keywords:
Cumulus
Oocyte
Prostaglandin E2
Protein kinase B

ABSTRACT

The production of prostaglandin E2 (PGE2) seems to play an important role in the ovulation process. PGE2 was found to induce cumulus expansion and meiosis resumption in mice, but little is known about its role in pigs. The goals of this study were (a) to assess the effect of PGE2 on the expression levels of cumulus expansion-related genes, (b) to define the signaling pathways that drive the PGE2-stimulated expression of cumulus expansion-related genes, (c) to measure the effect of PGE2 on the activation of key signaling molecules (MAPK3/1, PKB) and on hyaluronan production in cumulus cells, and (d) to assess the effect of PGE2 on meiosis resumption. We documented that PGE2 is able to induce the expression of cumulus expansion-related genes (*HAS2*, *TNFAIP6*) as well as genes involved in steroidogenesis (*CYP11A1*) or prostaglandin production (*PTGS2*). PGE2 is able to activate PKB and MAPK3/1 and induce mild cumulus expansion and meiosis resumption, but less efficiently than FSH.

1. Introduction

Cumulus cell expansion is a process that enables the detachment of the cumulus-oocyte complex (COC) from the follicle wall and subsequent ovulation. The process is accompanied by an extensive rearrangement of the cumulus cell cytoskeleton [1] and the production of a large amount of hyaluronic acid-rich extracellular matrix (ECM), which is synthetized by hyaluronan synthase 2 (HAS2). The organization of linear molecules of hyaluronan is mediated by tumor necrosis factor alpha induced protein 6 (TNFAIP6) [2] and pentraxin 3 [3]. Disruption of either of these genes leads to female infertility and has a large impact on the stability of the expanded cumulus and on hyaluronan retention. Hyaluronan binds to its receptor CD44 on the cumulus cell surface. Activation of CD44 by hyaluronan binding is able to inhibit apoptosis of human mural granulosa and cumulus cells [4]. In pigs, interaction of hyaluronan and CD44 seems to regulate meiosis resumption [5] and expression of *CD44* increases in a manner dependent on the degree of cumulus expansion [6]. Additionally, activity of the extracellular metalloprotease ADAMTS1 is required for the structural remodeling of the cumulus ECM and subsequent ovulation [7]. Interestingly, murine COCs expanded *in vitro* neither contain detectable ADAMTS1, nor intact or ADAMTS1-cleaved versican, a substrate of ADAMTS1 [8]. The altered composition of cumulus ECM and cumulus physiology may contribute to the lower developmental competence of oocytes cultivated *in vitro* [8,9]. Connection between ECM and cumulus cell seems to be mediated via syndecan 4 (SDC4). SDC4 is a heparan sulfate proteoglycan which serves as a direct link between the ECM and intracellular signaling proteins and cytoskeleton. Expression of *SDC4* in porcine cumulus cells is upregulated by follicle-stimulating hormone (FSH) [10] and its expression in human cumulus cells positively correlates with developmental competence of oocyte [11].

In response to the preovulatory surge of luteinizing hormone (LH), mural granulosa cells and cumulus cells start to produce EGF-like peptides amphiregulin (AREG) and epiregulin (EREG), which act as the mediators of LH action in the follicle [12,13]. Both *in vivo* and *in vitro*, the peptides are able to induce the resumption of meiosis and expansion of cumulus cells [12,13]. The peptides bind to epidermal growth factor receptor (EGFR) and induce activation of mitogen-activated protein kinases 3/1 (MAPK3/1), signaling molecules essential for cumulus

* Corresponding author.
Email address: blaha@iapg.cas.cz (M. Blaha)

http://dx.doi.org/10.1016/j.prostaglandins.2017.04.001
Received 6 December 2016; Received in revised form 17 March 2017; Accepted 3 April 2017
Available online xxx
1098-8823/

expansion and female fertility in mice [14] and pigs [15]. Targeted disruption of the gene coding CCAAT/enhancer-binding protein beta (*Cebpb*) in murine granulosa cells leads to the similar phenotype observed in granulosa-specific *Mapk3/1* knockout mice, indicating a role of CEBPB transcription factor in expression of cumulus expansion related-genes [14]. Both FSH and EGF-like peptides are able to activate protein kinase B (PKB) which seems to be required for FSH- and AREG-induced expression of *HAS2* and *TNFAIP6* [15]. Additionally, EGF-like peptides and FSH upregulate the expression of prostaglandin-endoperoxide synthase 2 (PTGS2), the rate-limiting enzyme in prostaglandin synthesis. The production of prostaglandins, especially prostaglandin E2 (PGE2), seems to be essential for female fertility. In mice, the targeted disruption of *Ptgs2* leads to severe disruption of ovulation and fertilization [16] and a similar phenotype was observed in mice lacking the receptor of prostaglandin E2 *Ptger2* [17]. Interestingly, the removal of cumulus cells from wild-type oocytes led to similar fertilization rates to those observed in *Ptger2* null oocytes (49% vs. 39%), indicating the importance of PGE2/PTGER2 for cumulus cell function [17,18]. Indeed, PGE2 induces cumulus expansion in many mammalian species, e.g. in mice [19–21], cows [22,23] or macaques [24]. PGE2 was found to upregulate the expression of key genes associated with cumulus expansion, namely *Areg* and *Ereg* in both mouse [25] and human granulosa cells [26], in addition to *Has2*, *Ptgs2* and *Tnfaip6* in mouse granulosa cells [25]. Interestingly, PGE2 is probably involved in the regulation of the cumulus ECM assembly via the expression of chemokines (*Ccl2*, *Ccl7* and *Ccl9*) in murine cumulus cells [27]. This prostaglandin is required for the downregulation of *Ccl7* expression, and thus PGE2 prevents excessive integrin engagement to the cumulus ECM, which causes fertilization failure [27]. Moreover, mouse cumuli expanded *in vivo* are able to retain cumulus cell-synthesized PGE2 within the complexes more than *in vitro*-expanded COCs stimulated with FSH and epidermal growth factor [9]. Although the causality is unclear, COCs expanded *in vitro* exhibit altered molecular filtration properties, which affects metabolite diffusion into cumuli and oocytes [9]. The correlation between the PGE2 receptor PTGER1 and SERPINE1 expression in non-apex cells indicates an involvement of PGE2 in the regulation of proteolytic events before stigma formation [28]. The role of PGE2 in cumulus expansion and stigma formation was also confirmed in *Pgs2* null mice [20].

The aim of this study was to describe the effect of PGE2-enriched medium on the expression of key genes involved in cumulus expansion in porcine COCs. Using pharmacological inhibitors of selected signaling proteins, we studied the signaling pathways which seem to mediate the effect of PGE2 on gene expression. We also quantified the concentration of PGE2 produced into the cultivation medium by FSH-stimulated COCs and assessed the effect of exogenous PGE2 on cumulus expansion.

2. Materials and methods

2.1. Culture media and reagents

All chemicals were purchased from Sigma-Aldrich (Prague, Czech Republic) unless otherwise specified.

2.2. Isolation and culture of cumulus-oocyte complexes

Ovaries of prepubertal gilts were collected at a local abattoir and immediately transported to the laboratory in a thermo-flask at 37 °C. COCs were aspirated from medium-sized antral follicles about 3–5 mm in diameter, washed in phosphate buffered saline (PBS) and cultured in M-199 medium (Gibco, Life Technologies, Rockville, MD, USA) supplemented with 0.91 mM sodium pyruvate, 0.57 mM cysteine, 5.5 mM Hepes, antibiotics and fetal calf serum (5%). Groups of 30 COCs were cultured in four-well dishes (Nunclon, Roskilde, Denmark) in 0.5 ml of cultivation media at 38.5 °C in a humidified atmosphere of 5% CO_2. PGE2 was dissolved in absolute ethanol and the stock solution (1 mg/ml) was stored at 4 °C. Shortly before use, 2 μl of stock solution was dissolved in 998 μl of cultivation medium and the solution was vortexed vigorously. Then 10 or 50 μl of this solution was added to a culture well to obtain a final concentration of PGE2 40 or 200 ng/ml. The maximum final concentration of ethanol in culture medium was 0.02% (v/v). For induction of PGE2 and hyaluronan production, FSH from sheep pituitary (1 IU/ml) was used. For the inhibition of protein kinase A (PKA), phosphoinositide 3-kinase/protein kinase B (PI3K/PKB), mitogen-activated protein kinases 1/3 (MAPK3/1), mitogen-activated protein kinase 14 (MAPK14) and epidermal growth factor receptor (EGFR) tyrosine kinase, the COCs were first exposed for 1 h to different concentrations of H89 (20 μM), LY294002 (25 μM), U0126 (10 μM; Merck Chemicals Ltd., Nottingham, UK), SB203580 (20 μM; Merck Chemicals Ltd., Nottingham, UK) or AG1478 (10 μM) respectively and then PGE2 was added (200 ng/ml). The inhibitors were dissolved in dimethyl sulfoxide (DMSO) and 10 or 100 mM stocks were stored frozen at −20 °C for a maximum period of 3 months. The maximum final concentration of DMSO in culture medium was 0.2% (v/v).

2.3. Assessment of oocyte maturation and cumulus cell expansion

To assess their nuclear maturation, oocytes were cultured for 42 h and then removed from cumulus cells by vortexing, mounted on slides and fixed in acetic ethanol for 48 h. Oocytes were then stained with 1% orcein and observed with a light microscope. Oocytes were scored for GV, GVBD (mostly comprised of oocytes at the MI stage and a few oocytes in late diakinesis, anaphase I or telophase I) and for the MII stage. The degree of cumulus expansion was assessed at 28 h after the onset of culture using a subjective scoring method [29]. Briefly, no response is scored as 0, minimal observable response, the cells in outermost layer of the cumulus become round and glistening as 1, the expansion of outer COCs layers as 2, the expansion of all COCs layers except *corona radiata* as 3 and the expansion of all COCs layers as 4. Afterwards, a cumulus expansion index (CEI; 0–4) was calculated as an average degree of expansion in the experimental group of COCs.

2.4. Real-time reverse transcription-polymerase chain reaction

The total RNA from 30 COCs cultured for 1, 4 and 8 h was isolated using an RNeasy Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. Real-time RT-PCR was carried out in a RotorGene 3000 cycler (Corbett Research, Sydney, Australia) using a One-Step RT-PCR Kit (Qiagen) with gene-specific primers shown in Table 1. The 25 μl total reaction volume contained QIAGEN OneStep RT-PCR Buffer (1×), dNTP Mix (400 μM final concentration of each), reverse and forward primers (both 400 nM final concentration), SybrGreenI (0.5 μl of 1:1000 stock solution, Molecular Probes, Eugene, Oregon), RNasine inhibitor (5 IU, Promega, Madison, WI, USA), QIAGEN OneStep RT-PCR Enzyme Mix (1 μl) and template RNA (3 μl). The reaction conditions were as follows: reverse transcription at 50 °C for 30 min, predenaturation at 95 °C for 15 min, followed by various numbers of PCR cycles, each of which consisted of denaturation at 95 °C for 30 s, annealing at a specific temperature for each pair of primers (shown in Table 1) for 20 s, extension at 72 °C for 30 s, and a final extension step at 72 °C for 5 min. The specificity of the PCR product was verified by melting analysis. The relative concentrations of templates in different samples were determined using comparative analysis software (Corbett Research). The results for individual target genes were normalized according to the relative concentration of the internal standard, *HPRT*.

Table 1
Primers used for real-time RT-PCR.

Gene transcript	Sequence 5′- 3′	Amplicon length [bp]	T_{an} [°C]	Gene Accesion Number
AREG	F: CTA TTG CTG TTG TTA TTA C R: GTT CTG TCT TCT TAT GAT	221	53	NM_214376
CD44	F: GAG GCG GCC CTG AAC ATA R: AAG GTA TTA GGC AGG TCT GTG AC	218	58	XM_013994409
CEBPB	F: TAC AAG ATC CGG CGT GAG R: CAG CTG CTT GAA CAA GTT CC	183	57	AB569088
CYP11A1	F: ATA CCT CGT GAA TGA CTT R: CCT GGA TTT GAG AAG AAG	111	53	NM_214427
EREG	F: AAG ACA ATC CAG GTG TGG CTC AAG R: CGA TTT TTG TAC CAT CTG CAG AAA	277	58	XM_013978775
HAS2	F: GAA GTC ATG GGC AGG GAC AAT TC R: TGG CAG GCC CTT TCT ATG TTA	407	54	NM_214053
HPRT1	F: CCA GTA AAC GGG CGA TAT AA R: CTT GAC CAA GGA AAG CAA GG	129	55	NM_001032376
PTGS2	F: TCG ACC AGA GCA GAG AGA TGA GAT R: ACC ATA GAG CGC TTC TAA CTC TGC	260	55	NM_214321
SDC4	F: GGC AGC AAC ATC TTC GAG AG R: GGT TTC TTG CCC AGG TCG TA	149	58	NM_214284
TNFAIP6	F: CAG AAG ACA TCA TTA GTA R: CAG TAG AAG TAG TAG TTG	150	54	NM_001159607

T_{an} – annealing temperature.

2.5. Immunoblotting

At the end of each culture, COCs were washed in PBS and solubilized in Laemmli buffer containing 2% sodium dodecyl sulphate (SDS) and 5% 2-mercaptoethanol. Samples were boiled at 100 °C for 3 min and frozen (−20 °C). Subsequently, proteins were separated in 10% acrylamide/SDS gels and transferred to Immobilon-P membranes (Millipore, Bedford, MA, USA). Membranes were blocked in 5% low fat dry milk in Tris-buffered saline (TBS) with 0.5% Tween 20 for 2 h at room temperature and then incubated with the primary antibody diluted 1:1000 in 5% BSA in TBS-Tween, at 4 °C overnight. The primary antibodies were phospho-AKT (Ser 473), AKT (detecting PKB) – both from Cell Signaling Technology, and p-ERK, ERK (detecting MAPK3/1) – both from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The secondary antibodies (anti-mouse or anti-rabbit IgG conjugated with HRP; GE Healthcare, Little Chalfont, UK) were diluted 1:5000 in 2% BSA in TBS-Tween. The membranes were incubated with the secondary antibody for 1 h at room temperature and washed intensively in TBS-Tween. The immune reaction was detected by enhanced chemiluminescence (Pierce, Rockford, IL, USA) according to the manufacturer's instructions. The intensity of the specific bands on the blots was analysed by scanning densitometry using the free software ImageJ Version 1.29 (National Institute of Mental Health, Bethesda, MD, USA).

2.6. Measurement of PGE2 concentration in cultivation media

To quantify the concentration of PGE2 produced by COCs into the cultivation media, 30 COCs were cultivated in 500 µl of maturation medium for various periods of time (1, 4, 8, 12, 24, 48 h) in the presence or absence of FSH (1 IU/ml). The cultivation media were aspirated at the end of the culture and stored at −20 °C. The PGE2 concentrations in the samples were determined using a PGE2 ELISA kit (Enzo Life Sciences, Famingdale, NY, USA) according to the manufacturer's instructions. Briefly, 100 µl of PGE2 standards or samples were added to wells coated with affinity-purified antibody. A blue solution of PGE2 conjugated to alkaline phosphatase (ALP) was then added, followed by a yellow solution of monoclonal antibody to PGE2. The plate was incubated for 2 h on a shaker at room temperature to promote the immune reaction of endogenous and ALP-conjugated exogenous PGE2 with the antibody and binding of the complex to the affinity antibody. The wells were emptied after incubation and extensively washed three times with the supplied wash-buffer to leave only bound PGE2 in the wells. A total of 200 µl of *p*-nitrophenyl phosphate solution, an alkaline phosphatase substrate, was then added to each well and the plate was incubated for 1 h at room temperature in the dark without shaking. The reaction was stopped with trisodium phosphate solution and the amount of signal,

proportional to the amount of PGE2 in the sample, was measured with an ELISA plate reader (Synergy HT, BioTek, Winooski, VT, USA) at 405 nm. The PGE2 concentrations in the samples were calculated from a standard curve and are expressed as pg/ml.

2.7. Hyaluronan retention assay

Groups of 30 COCs were cultured for 24 h in 500 μl of cultivation medium with FSH (1 IU/ml) or PGE2. At the end of the culture period, the COCs were washed four times in 500 μl PBS-PVA. To determine the HA retained in the expanded cumuli, the last washing was quantitatively transferred into an Eppendorf tube and stored at −20 °C until use. Subsequently, the samples were enzymatically digested with the lyase from Streptomyces hyalurolyticus (2 IU/ml) at 39 °C overnight. The solutions were spectrophotometrically measured using Helios Gamma (Fisher Scientific, USA) at 216 nm against a blank consisting of PBS-PVA. The quadratic calibration curve was based on five hyaluronan standards (0.006–0.1% sodium hyaluronate) diluted and digested according to the protocol used for samples. The amount of hyaluronan obtained by lysis of the expanded cumuli is expressed in μg/ml of the measured sample. Comparison with expanded COCs classification or expansion area revealed the used method of hyaluronan quantification as suitable for an evaluation of cumulus expansion *in vitro* [30].

2.8. Statistical analysis

The statistical analyses were performed with the software GraphPad Prism 5.0 (La Jolla, CA, USA). Each experiment was performed in at least 3 replicates. Each replicate was carried out on a different day on a single batch of COCs that were randomly allocated to the treatments. The differences in the percentages of maturing oocytes, cumulus expansion index, PGE2-induced gene expression and the densitometrical quantifications of MAPK3/1 were compared by analysis of variance (ANOVA) followed by Tukey's post-test. The differences in concentration of hyaluronan in samples of COCs and the effects of inhibitors on PGE2-induced gene expression were analysed by unpaired *t*-test. The Kolmorogov-Smirnov test was used to examine the normal distribution of all data. A level of $P < 0.05$ was considered significant. Error bars indicate the standard error of the mean (SEM).

3. Results

3.1. PGE2 upregulates the expression of key genes involved in cumulus cell function

In order to determine the effect of PGE2 on gene expression in cumulus cells, COCs were cultured for 1, 4 or 8 h in two different concentrations of PGE2 (40 or 200 ng/ml). PGE2 was found to induce the expression of EGF-like factors aphiregulin (*AREG*) and epiregulin (*EREG*). The PGE2-treatment also upregulated the expression of the key transcription factor *CEBPB* and genes associated with ECM production and organization (*CD44, HAS2*, *TNFAIP6*, *SDC4*) and steroid hormone (*CYP11A1*) and prostaglandin (*PTGS2*) synthesis (Fig. 1).

3.2. PGE2-induced expression of key genes is downregulated by inhibitors of multiple signaling proteins

Subsequently, we focused on the signaling proteins which may participate in the regulation of the PGE2-induced expression of *AREG*, *EREG*, *HAS2*, *PTGS2* and *TNFAIP6*. As shown in Fig. 1, the expression levels of *AREG*, *EREG*, and *HAS2* peak at 4 h of culture. Based on these results, we chose 4 h interval as optimal for all selected genes. The inhibitors of MAPK3/1 (U0126), MAPK14 (SB203580), and the EGFR (AG1478) were found to downregulate the PGE2-induced expression of all studied genes. Interestingly, an inhibitor of phosphoinositide 3-kinase (LY294002) dramatically reduced the expression of *HAS2* and *TNFAIP6*, but upregulated on the *PTGS2* expression level (Fig. 2). Moreover, LY294002 significantly increased the expression of *AREG* in COCs cultivated with PGE2. Surprisingly, the inhibitor of PKA (H89) downregulated the expression levels of all studied genes except of *AREG*.

3.3. PGE2 activates protein kinase B and MAPK3/1 in COCs

The inhibitors of MAPK3/1 and PKB had disrupted PGE2-induced expression of cumulus expansion-related genes and EGF-like factors and these results led us to a hypothesis that PGE2 itself is able to activate PKB and MAPK3/1. We examined the activity of PKB and MAPK3/1 in COCs cultivated in PGE2-enriched medium for various periods of time. We found that PGE2 promptly (within 10 min) activated both PKB and MAPK3/1, which was documented by a significant increase in phosphorylated variants of the kinases over the level detected in control samples collected at the beginning of the culture (Fig. 3A). The increased activity of MAPK3/1 returned to the base level in 0.5–1.0 h (Fig. 3B) and the activity of PKB in 2 h (Fig. 3C). No increase in the activity of the kinases occurred during the culture of COCs in control medium without PGE2 (data not shown).

3.4. The effect of PGE2 on oocyte in vitro maturation and cumulus cell expansion

In control medium without PGE2 or FSH (1 IU/ml), approximately 71% of oocytes remained in the GV stage, 16% underwent GVBD and 13% of oocytes reached metaphase II during the culture period of 42 h (Table 2). The addition of 200 ng/ml of PGE2 to the culture medium resulted in a significant decrease in the proportion of oocytes remaining in the GV stage (47%; $P < 0.05$) and in a significant increase in the proportion of oocytes reaching MII (30%; $P < 0.05$). However, the PGE2 was less efficient at stimulating oocyte maturation than FSH (1 IU/ml), which induced the completion of meiosis to the MII stage in 84% of oocytes ($P < 0.001$ for FSH vs. control and FSH vs. PGE2).

The expansion of cumulus cells was not stimulated in COCs cultured in the control medium (Table 2; Fig. 4A). The addition of PGE2 resulted in a significant and dose-dependent increase in CEI. However, the expansion reached degree 1 in most COCs and about 20% of COCs displayed degree 2 or 3 (Fig. 4B). In contrast, FSH (1 IU/ml) induced degree 3 or 4 in the vast majority of the cultured COCs (Fig. 4C).

3.5. PGE2 production into the cultivation media

To quantify the concentration of PGE2 produced by COCs cultivated in the presence of FSH (1 IU/ml), 30 COCs were cultivated in 500 μl of maturation medium for various periods of time (1, 4, 8, 12, 24, 48 h) with or without FSH. The highest concentration of PGE2 was observed at 24 h (average 2917 pg/ml ≈ 8276 pmol/l), indicating that one COC was able to produce approximately 49 pg (0.14 pmol) of PGE2. At 48 h of cultivation, the PGE2 concentration decreased dramatically (Fig. 5).

3.6. Hyaluronan production and retention in cumulus

A spectrophotometric assay was used to evaluate the hyaluronan production and retention in the cumuli of COCs cultivated with FSH (1 IU/ml) or PGE2 for 28 h. Using this approach, we found a significant increase in the amount of hyaluronan in COCs cultivated with FSH and higher concentration of PGE2 (200 ng/ml) compared to control COCs (cultivated neither with FSH nor PGE2). On the other hand, no statistically significant differences were observed between control COCs and COCs stimulated by lower concentration of PGE2 (40 ng/ml) (Fig. 6).

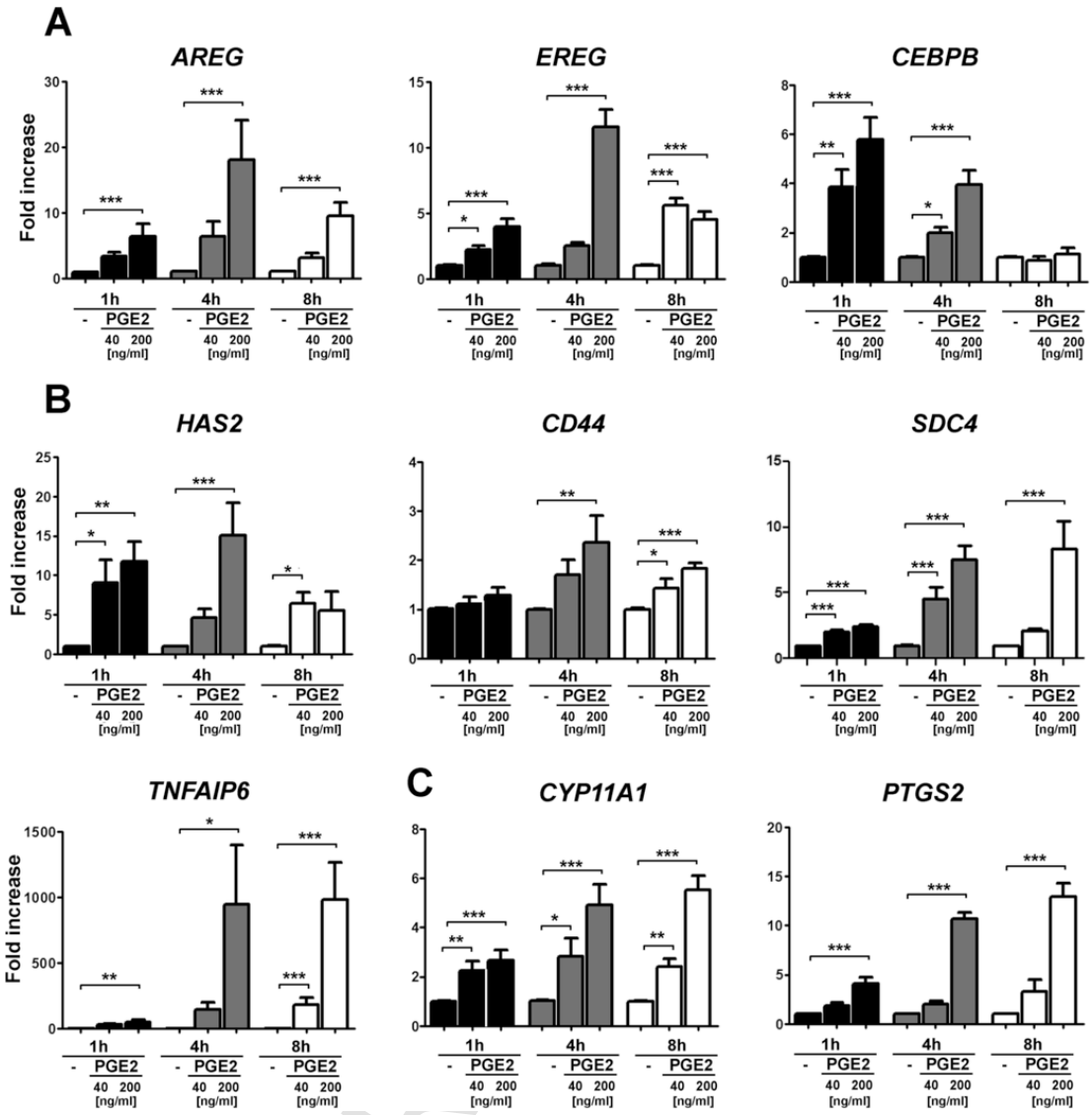


Fig. 1. Effect of PGE2 on the expression of genes in COCs cultured for 1, 4 and 8 h. A. Genes encoding signaling molecules. B. Genes involved in hyaluronan synthesis and organization of ECM. C. Genes involved in regulation of follicle steroidogenesis and PGE2 production. The relative concentrations of the template in the different samples were determined using comparative analysis software (Corbett Research). The relative abundance of specific gene mRNA is expressed in arbitrary units as fold increases in the specific gene/*HPRT* ratio over the level found in the control group of COCs cultured for the depicted intervals in medium without PGE2. The data were analysed by the ANOVA followed by Tukey's post-test, for each time interval separately. The values with asterisks are significantly different from the corresponding value of the control group within the same period of culture. $P < 0.05$ for single asterisk; $P < 0.01$ for double asterisk; $P < 0.001$ for triple asterisk.

4. Discussion

4.1. PGE2 concentration

In our preliminary experiments, we found that the expression of *HAS2* and *TNFAIP6* was stimulated by PGE2 at concentrations of at least 40 ng/ml (data not shown). A similar concentration of PGE2 (35 ng/ml) stimulates the expression of *AREG* and *EREG* in human granulosa cells [26]. In cattle, a COC cultivated with estradiol, FSH and LH for 24 h produces 0.7 ± 0.3 ng PGE2 [31], which gives almost the same concentration (42 ng/ml) as in our cultivation system (30 COCs in 500 μl of medium). The concentration of PGE2 in follicular fluid in bovine follicles found 24 h after the onset of estrus was 87.9 ± 30.9 ng/ml [32]. These data indicate that concentrations of PGE2 used in this study are in the relevant physiological range. In mice, the stimulatory effect of PGE2 on cumulus expansion is well documented by many studies. PGE2 was found to stimulate the production of hyaluronan at similar concentrations [19,33] to those used in our study. We also documented that porcine COCs cultivated with FSH produce significant amounts of PGE2 into the cultivation media (max. 2.9 ng/ml). A comparable concentration of PGE2 (up to 10 ng/ml) was produced by murine COCs stimulated with FSH [21]. On the other hand, previously published data indicate that porcine COCs stimulated with FSH are only able to produce about 0.5 ng/ml of PGE2 [34]. However, both studies [21,34] suggest that the maximal concentration of PGE2 is reached at 18–20 h of culture, which is consistent with the data presented in this study. The dramatic decrease in PGE2 concentration at 48 h may be a result of dynamic equilibrium between PGE2 production and its degradation. It is very difficult to estimate PGE2 half-life/stability in our cultivation system, but PGE2 half-life seems to be short as indicate *in vivo* studies and paracrine/autocrine mechanism of action. In cats, the half-time of PGE2 injected intravenously is 0.29–1.08 min [35]. On the other hand, it is very difficult to compare

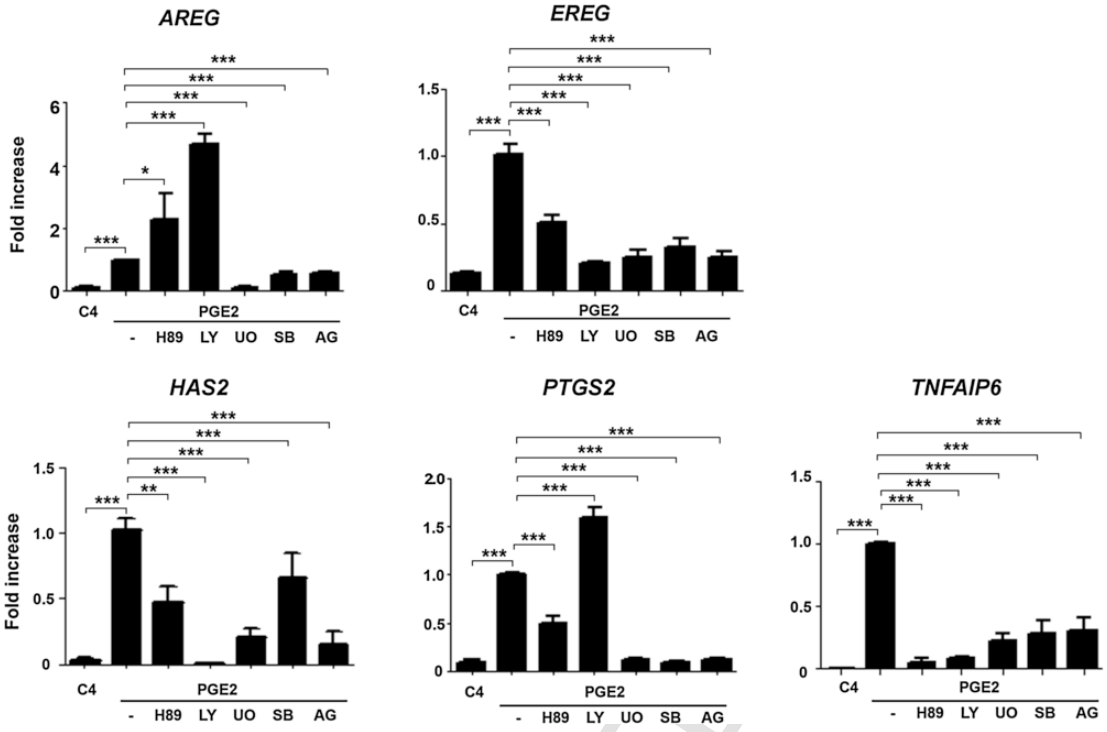


Fig. 2. Effect of signaling protein inhibitors on PGE2-stimulated expression of genes in COCs. To define signaling pathways that drive PGE2-induced gene expression, porcine COCs were treated for 1 h with inhibitor of PKA (H89), PI3K/PKB (LY294002), MAPK3/1 (U0126), MAPK14 (SB203580) or EGFR (AG1478) respectively and then PGE2 was added (200 ng/ml). C4 represents COCs cultivated in PGE2-free medium for 4 h. The relative concentrations of the template in the different samples were determined using comparative analysis software (Corbett Research). The relative abundance of specific gene mRNA is expressed in arbitrary units as fold increases in the specific gene/*HPRT* ratio over the level found in the group of COCs cultured with PGE2 for 4 h. The data were analysed by unpaired *t*-test. The Kolmorogov-Smirnov test was used to examine normal distribution of all data. The values with asterisks are significantly different from the corresponding value of the group of COCs cultured with PGE2 without inhibitors. $P < 0.05$ for single asterisk; $P < 0.01$ for double asterisk; $P < 0.001$ for triple asterisk.

in vivo and *in vitro* PGE2 elimination, because *in vivo* half-time is short due to extensive pulmonary inactivation of the prostaglandin. PGE2 is probably more stable *in vitro* than *in vivo* but there are many factors which may affect its *in vitro* degradation, e. g. albumin present in culture medium. Moreover, activity of 15-hydroxyprostaglandin dehydrogenase, an enzyme responsible for PGE2 biological inactivation, was found to be upregulated by gonadotropin stimulation in macaque granulosa cells [36].

4.2. Gene expression

The first aim of our study was to describe the effect of a PGE2-enriched medium on the expression of key genes involved in cumulus expansion in porcine COCs. We have shown previously that exposure of COCs to FSH as brief as 3 h, followed by culture in hormone-free medium, is sufficient to initiate cumulus expansion and the resumption of pig oocyte meiosis [37]. Additionally, the expression levels of some key genes seem to be upregulated within one hour after gonadotropin addition [10] and the expression of some regulatory genes became downregulated by 4 h after FSH addition [10,13]. Based on this background, we chose the specific time intervals 1, 4 and 8 h.

We documented a significant upregulation of the expression of genes coding precursors of the EGF-like peptides AREG and EREG, which are the mediators of LH signaling in mice [12] and pigs [13]. PGE2 probably promotes the expression or release of EGF-like factors which are able to upregulate *PTGS2* expression and thus the AREG/PGE2 system represents a positive feedback loop as previously proposed [25]. Moreover, very prompt phosphorylation of MAPK3/1 and PKB indicates, that PGE2 itself is able to activate both these key kinases without previous upregulation of *AREG* and *EREG* expression. On the other hand, the activity of PTGS2 seems to be non-essential for the expression of *AREG*, *EREG*, *HAS2*, and *TNFAIP6* during the first hours of *in vitro* cultivation of porcine COCs stimulated with FSH [34]. These results can be explained by the fact that FSH itself is also able to activate MAPK3/1 and PKB as previously reported in rat granulosa cells [38].

PGE2 also induced the expression of *CEBPB*, which was found to be essential for the expression of cumulus expansion-related genes [14]. Our data support a previously published conclusion that CEBPA and CEBPB mediate specific events downstream of both cAMP/PKA and MAPK3/1 [39]. In our previous study, *CEBPB* expression was found to be upregulated more effectively by FSH than by EGF-like peptides [10]. Data presented in this study indicate that *CEBPB* is upregulated by PGE2 more promptly than by FSH.

Our data and data from other groups strongly suggest that PGE2 is especially crucial for *Tnfaip6* expression. In ovarian follicles of *Ptgs2*$^{-/-}$ and *Ptger2*$^{-/-}$ mice stimulated with human chorionic gonadotropin, the expression of *Tnfaip6* is absent in cumulus cells [40]. Moreover, PGE2 is able to restore cumulus expansion and TNFAIP6 expression in COCs obtained from *Ptgs2*$^{-/-}$ mice [21].

4.3. Signaling pathways

To define the signaling pathways that drive the PGE2-induced expression of cumulus expansion-related genes, we used a panel of pharmacological inhibitors of selected signaling molecules. In accordance with our previous results [15], inhibitors of MAPK3/1 and EGFR caused a significant decrease in the PGE2-induced expression of all selected genes, which confirms the essential role of MAPK3/1 and EGFR in the process of cumulus expansion [14]. The inhibitor of MAPK14 also dramatically reduced the expression of *AREG, EREG, HAS2*, *PTGS2* and *TNFAIP6*. These results support the conclusions of the study of

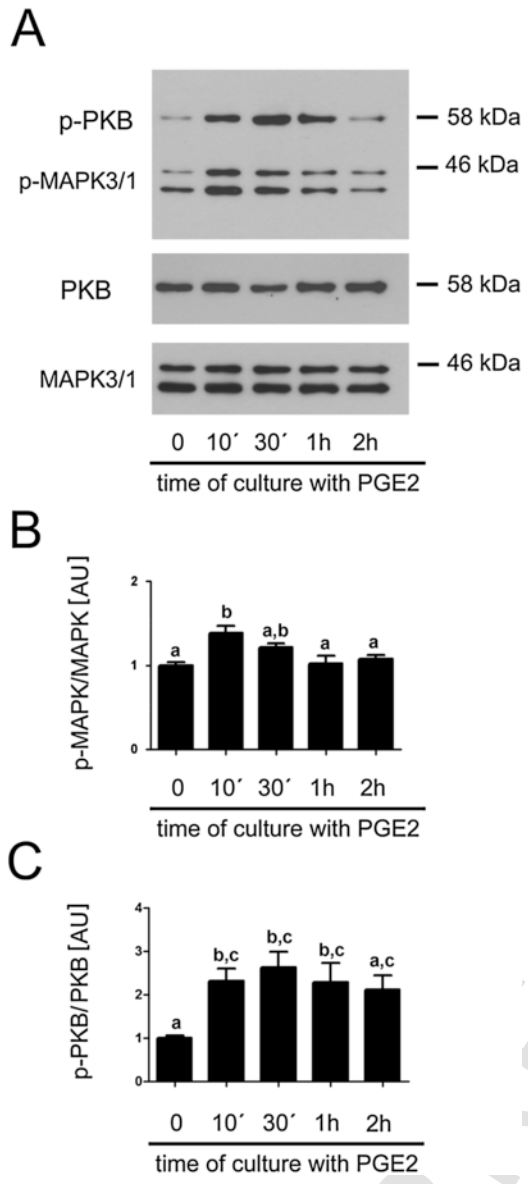


Fig. 3. Effect of PGE2 (200 ng/ml) on activation of PKB and MAPK3/1. A. A representative result of immunoblotting of phosphorylated PKB and MAPK3/1 (top panel) and total PKB and MAPK3/1 (middle and bottom panels) in samples of 25 COCs cultivated *in vitro* for the indicated periods of time. B. Quantification of the activated MAPK3/1 by densitometry. The results are shown as proportions of the phosphorylated and total MAPK3/1 and expressed in arbitrary units as a fold increase over the proportion found in COCs at the beginning of the cultivation. The data were analysed by the ANOVA followed by Tukey's post-test. The different superscripts above the columns indicate significant differences ($P < 0.05$). The data were summarized from 5 independent experiments. C. Quantification of activated PKB by densitometry. The results are shown as proportions of the phosphorylated PKB and total PKB and expressed in arbitrary units as a fold increase over the proportion found in COCs at the beginning of the culture. The data were analysed by the ANOVA followed by Tukey's post-test. The different superscripts above the columns indicate significant differences ($P < 0.05$). The data were summarized from 5 independent experiments.

granulosa-specific *Mapk14* null mice (*Mapk14*$^{gc-/-}$). In the COCs of these animals, PGE2 neither induces cumulus expansion nor the expression of *Areg*, *Has2* and *Ptgs2* [25]. Our previously published results also confirm the importance of MAPK14 for both the AREG- and FSH-induced expression of cumulus expansion-related genes [15]. Surprisingly, the inhibitor of PI3K/PKB dramatically reduced the expression levels of *HAS2* and *TNFAIP6*, but upregulated *PTGS2* expression. These results are in accordance with our previous finding that the PI3K inhibitor decreases the FSH- or AREG-induced expression of *HAS2* and *TNFAIP6*, but upregulates *PTGS2* mRNA level [15]. These data led us to a hypothesis that the increase in *PTGS2* expression may represent a compensatory mechanism to maintain PKB activity. To test the hypothesis, we studied the effect of PGE2 on PKB phosphorylation in both intact COCs and oocytes. In accordance with previously published findings in mice [21], we confirmed a prompt and transient PKB activation in porcine COCs within 1 h of PGE2 addition to the cultivation medium. Moreover, PGE2 was found to activate MAPK3/1, the signaling molecules essential for cumulus expansion in mice [14] and pigs [15]. These data indicate that PGE2-induced activation of MAP3/1 or PKB is not dependent on induction of *AREG* and *EREG*.

4.4. Hyaluronan production and retention

Despite the activation of key signaling molecules and expression of cumulus expansion-related genes, our results indicate that PGE2 itself is not able to induce full cumulus expansion in porcine COCs. Similarly in bovine COCs, PGE2 induced only mild cumulus expansion, even at a concentration of 1000 ng/ml [23]. To objectify this observation, we measured the amount of hyaluronan retained in cumuli from COCs cultivated with FSH (1 IU/ml) or PGE2. No statistically significant differences were observed between control COCs and COCs stimulated by lower concentration of PGE2 (40 ng/ml). Higher PGE2 concentration (200 ng/ml) induced significant increase in hyaluronan production/retention, but still relatively low compared to FSH-stimulated COCs, indicating the absence of factors essential for hyaluronan synthesis or retention in the cumulus. In porcine cumuli, hyaluronan is bound to its receptor CD44, which is localized on the surface of cumulus cells [41]. *In vitro*, the expression of *CD44* in porcine cumulus cells was found to be upregulated by equine chorionic gonadotropin [41], and interaction with hyaluronan-CD44 seems to be required for meiosis resumption [5]. However, PGE2 was able to upregulate *CD44* expression in similar way as FSH (1 IU/ml, data not shown). Thus we suggest that a complex analysis of the expression profiles of PGE2- and FSH-stimulated COCs should be performed using a different experimental approach (e.g. microarrays).

4.5. Meiotic maturation

Our final aim was to assess the effect of PGE2 on meiotic maturation. PGE2 induced meiosis resumption, but less efficiently than FSH. Our data are in accordance with previously published experiments in mice [21]. On the other hand, PGE2 (above 50 ng/ml) was found to stimulate the meiotic maturation of bovine COCs as effectively as FSH + LH [23].

4.6. Conclusions

We documented that PGE2 added to the cultivation medium at a concentration higher than 40 ng/ml is able to induce the expression of cumulus expansion-related genes and mild cumulus expansion. The substance also promotes the resumption of meiosis but less efficiently than gonadotropins. PGE2 activates PKB and MAPK3/1 in cumulus cells, which are essential for cumulus-expansion gene expression. Activation of MAKP3/1 and PKB was observed within 10 min after PGE2 addition indicating that PGE2-induced expression of *AREG* and *EREG* is not required for such prompt activation. Activation of these key signaling molecules probably allows upregulation of genes previously associated to cumulus expansion and antral follicle development. Although MAPK14 is involved in PGE2-induced gene expression, we would like to point out the importance of PKB which is activated by PGE2. Upregulation of *PTGS2* expression in COCs cultivated with the inhibitor of PI3K indicates a possible compensatory mechanism to secure the PKB activity required for *HAS2* and *TNFAIP6* expression. Further research of

Table 2
Effect of prostaglandin E2 on maturation of pig oocytes and expansion of cumulus cells.

Treatment of COCs	No. of oocytes	% of oocytes in			Cumulus expansion
during maturation (42 h)	examined	GV	GVBD	MII	index
None	150	70.9 ± 4.2^{a}	16.1 ± 3.2	13.0 ± 3.8^{a}	0^{a}
PGE2 (40 ng/ml)	146	63.2 ± 8.0^{ab}	18.1 ± 3.6	18.7 ± 3.5^{ab}	1.03 ± 0.05^{b}
PGE2 (200 ng/ml)	186	47.1 ± 5.0^{b}	23.0 ± 4.0	29.9 ± 2.9^{b}	1.49 ± 0.05^{c}
FSH	139	5.7 ± 2.5^{c}	9.9 ± 1.9	84.4 ± 3.6^{c}	3.02 ± 0.03^{d}

The values are shown as means ± SEM. Values with no common letter in superscript are significantly different within a column ($P < 0.05$ for the maturation values and $P < 0.001$ for the cumulus expansion index). Data were obtained from 6 replicates. COCs: cumulus-oocyte complexes. GV: germinal vesicle. GVBD: germinal vesicle breakdown. MII: metaphase II. PGE2: prostaglandin E2. FSH: follicle stimulating hormone.

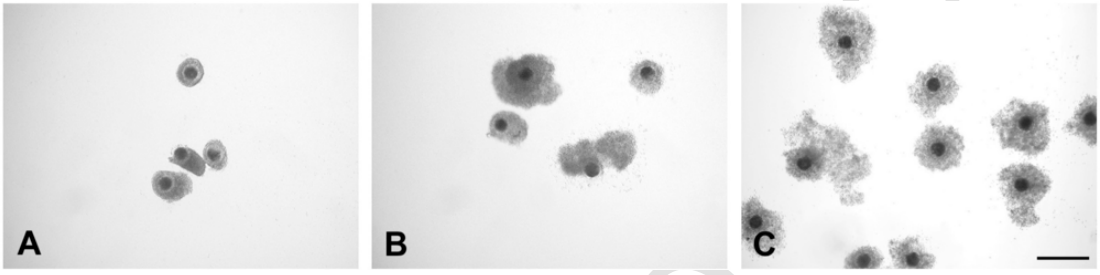


Fig. 4. Effect of PGE2 and FSH (1 IU/ml) on expansion of pig COCs. A. COCs cultivated for 28 h in control medium. B. COCs cultured in medium with 200 ng/ml PGE2. The image shows selected COCs that responded to the stimulation by expansion of the external layer of cumulus cells (degree 2; see Materials and methods). C. COCs cultivated in medium with FSH; almost all COCs display degree 3 of expansion. Bar = 400 μm.

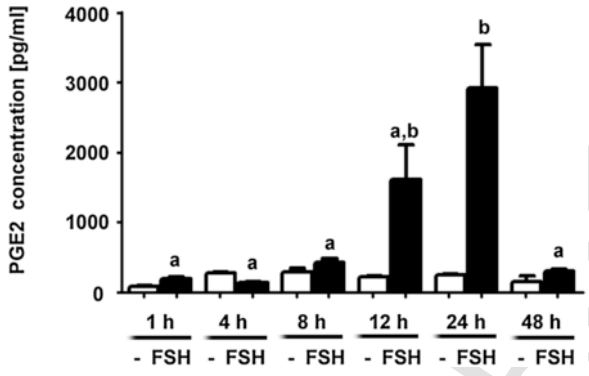


Fig. 5. Production of PGE2 by FSH-stimulated (1 IU/ml) COCs during in vitro cultivation. The data were analysed by the ANOVA followed by Tukey's post-test. The value with superscript is significantly different from the other values ($P < 0.05$).

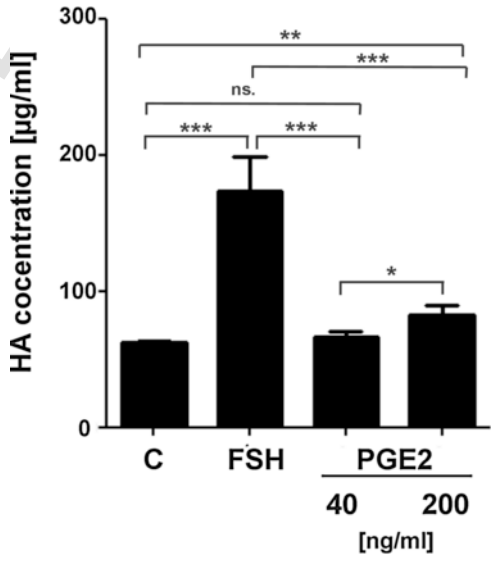


Fig. 6. Retention of hyaluronanic acid (HA) in COCs stimulated with FSH and PGE2. The data were analysed by unpaired *t*-test. The Kolmorogov-Smirnov test was used to examine normal distribution of all data. $P < 0.05$ for single asterisk; $P < 0.01$ for double asterisk; $P < 0.001$ for triple asterisk.

the role of PGE2 in oocyte maturation and cumulus expansion may provide new insights into cumulus cell function and the improvement of cultivation systems. Previous data from our [10,42] and other groups [43,44] indicate that the exposure of COCs to cAMP-elevating substances followed by cultivation with EGF-like factors and steroid hormones may have beneficial effect on oocyte developmental competence and cumulus cell function. PGE2 as a substance inducing cAMP production may be useful in new two-step cultivation systems.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

MB designed the experiments, isolated and cultured the COCs, conducted RT-PCR and ELISA, and wrote the manuscript. LN performed RT-PCR, analysed the data and revised the manuscript. RP performed immunoblotting and wrote the manuscript. JN and KA performed hyaluronan retention assay. All authors read and approved the final manuscript.

Acknowledgments

This work was supported by grant No. QJ1510138 from the National Agency for Agricultural Research and grant CZ.02.1.01/0.0/0.0/15_003/0000460 from the Operational Programme Research, Development and Education. Additional support was provided by Institutional Research Concept RVO 67985904 (IAPG, CAS). The authors would like to thank Mr B. J. Watson-Jones for reading the manuscript and language correction.

References

[1] R. Prochazka, V. Srsen, E. Nagyova, T. Miyano, J.E. Flechon, Developmental regulation of effect of epidermal growth factor on porcine oocyte-cumulus cell complexes: nuclear maturation, expansion, and F-actin remodeling, Mol. Reprod. Dev. 56 (2000) 63–73.

[2] C. Fülöp, S. Szántó, D. Mukhopadhyay, T. Bárdos, R.V. Kamath, M.S. Rugg, A.J. Day, A. Salustri, V.C. Hascall, T.T. Glant, K. Mikecz, Impaired cumulus mucification and female sterility in tumor necrosis factor-induced protein-6 deficient mice, Development 130 (2003) 2253–2261.

[3] A. Salustri, C. Garlanda, E. Hirsch, M. De Acetis, A. Maccagno, B. Bottazzi, A. Doni, A. Bastone, G. Mantovani, P. Beck Peccoz, G. Salvatori, D.J. Mahoney, A.J. Day, G. Siracusa, L. Romani, A. Mantovani, PTX3 plays a key role in the organization of the cumulus oophorus extracellular matrix and in in vivo fertilization, Development 131 (2004) 1577–1586.

[4] T. Kaneko, H. Saito, M. Toya, T. Satio, K. Nakahara, M. Hiroi, Hyaluronic acid inhibits apoptosis in granulosa cells via CD44, J. Assist. Reprod. Genet. 17 (2000) 162–167.

[5] M. Yokoo, N. Kimura, E. Sato, Induction of oocyte maturation by hyaluronan-CD44 interaction in pigs, J. Reprod. Dev. 56 (2010) 15–19.

[6] E. Sato, M. Yokoo, Morphological and biochemical dynamics of porcine cumulus-oocyte complexes: role of cumulus expansion in oocyte maturation, Ital. J. Anat. Embryol. 110 (2005) 205–217.

[7] H.M. Brown, K.R. Dunning, R.L. Robker, D. Boerboom, M. Pritchard, M. Lane, D.L. Russell, ADAMTS1 cleavage of versican mediates essential structural remodeling of the ovarian follicle and cumulus-oocyte matrix during ovulation in mice, Biol. Reprod. 83 (2010) 549–557.

[8] K.R. Dunning, M. Lane, H.M. Brown, C. Yeo, R.L. Robker, D.L. Russell, Altered composition of the cumulus-oocyte complex matrix during in vitro maturation of oocytes, Hum. Reprod. 22 (2007) 2842–2850.

[9] K.R. Dunning, L.N. Watson, D.J. Sharkey, H.M. Brown, R.J. Norman, J.G. Thompson, R.L. Robker, D.L. Russell, Molecular filtration properties of the mouse expanded cumulus matrix: controlled supply of metabolites and extracellular signals to cumulus cells and the oocyte, Biol. Reprod. 87 (2012) 89.

[10] M. Blaha, L. Nemcova, K.V. Kepkova, P. Vodicka, R. Prochazka, Gene expression analysis of pig cumulus-oocyte complexes stimulated in vitro with follicle stimulating hormone or epidermal growth factor-like peptides, Reprod. Biol. Endocrinol. 13 (2015) 113.

[11] S. Wathlet, T. Adriaenssens, I. Segers, G. Verheyen, H. Van de Velde, W. Coucke, R. Ron El, P. Devroey, J. Smitz, Cumulus cell gene expression predicts better cleavage-stage embryo or blastocyst development and pregnancy for ICSI patients, Hum. Reprod. 26 (2011) 1035–1051.

[12] J.Y. Park, Y.Q. Su, M. Ariga, E. Law, S.L. Jin, M. Conti, EGF-like growth factors as mediators of LH action in the ovulatory follicle, Science 303 (2004) 682–684.

[13] R. Prochazka, M. Petlach, E. Nagyova, L. Nemcova, Effect of epidermal growth factor-like peptides on pig cumulus cell expansion, oocyte maturation, and acquisition of developmental competence in vitro: comparison with gonadotropins, Reproduction 141 (2011) 425–435.

[14] H.Y. Fan, Z. Liu, M. Shimada, E. Sterneck, P.F. Johnson, S.M. Hedrick, J.S. Richards, MAPK3/1 (ERK1/2) in ovarian granulosa cells are essential for female fertility, Science 324 (2009) 938–941.

[15] R. Prochazka, M. Blaha, L. Nemcova, Signaling pathways regulating FSH- and amphiregulin-induced meiotic resumption and cumulus cell expansion in the pig, Reproduction 144 (2012) 535–546.

[16] H. Lim, B.C. Paria, S.K. Das, J.E. Dinchuk, R. Langenbach, J.M. Trzaskos, S.K. Dey, Multiple female reproductive failures in cyclooxygenase 2-deficient mice, Cell 91 (1997) 197–208.

[17] H. Hizaki, E. Segi, Y. Sugimoto, M. Hirose, T. Saji, F. Ushikubi, T. Matsuoka, Y. Noda, T. Tanaka, N. Yoshida, S. Narumiya, A. Ichikawa, Abortive expansion of the cumulus and impaired fertility in mice lacking the prostaglandin E receptor subtype EP(2), Proc. Natl. Acad. Sci. U. S. A. 96 (1999) 10501–10506.

[18] S.L. Tilley, L.P. Audoly, E.H. Hicks, H.S. Kim, P.J. Flannery, T.M. Coffman, B.H. Koller, Reproductive failure and reduced blood pressure in mice lacking the EP2 prostaglandin E2 receptor, J. Clin. Invest. 103 (1999) 1539–1545.

[19] J.J. Eppig, Prostaglandin E2 stimulates cumulus expansion and hyaluronic acid synthesis by cumuli oophori isolated from mice, Biol. Reprod. 25 (1981) 191–195.

[20] B.J. Davis, D.E. Lennard, C.A. Lee, H.F. Tiano, S.G. Morham, W.C. Wetsel, R. Langenbach, Anovulation in cyclooxygenase-2-deficient mice is restored by prostaglandin E2 and interleukin-1beta, Endocrinology 140 (1999) 2685–2695.

[21] T. Takahashi, J.D. Morrow, H. Wang, S.K. Dey, Cyclooxygenase-2-derived prostaglandin E(2) directs oocyte maturation by differentially influencing multiple signaling pathways, J. Biol. Chem. 281 (2006) 37117–37129.

[22] M.D. Calder, A.N. Caveney, M.E. Westhusin, A.J. Watson, Cyclooxygenase-2 and prostaglandin E(2)(PGE(2)) receptor messenger RNAs are affected by bovine oocyte maturation time and cumulus-oocyte complex quality, and PGE(2) induces moderate expansion of the bovine cumulus in vitro, Biol. Reprod. 65 (2001) 135–140.

[23] W.F. Marei, D.R. Abayasekara, D.C. Wathes, A.A. Fouladi-Nashta, Role of PTGS2-generated PGE2 during gonadotrophin-induced bovine oocyte maturation and cumulus cell expansion, Reprod. Biomed. Online 28 (2014) 388–400.

[24] M.C. Peluffo, J. Stanley, N. Braeuer, A. Rotgeri, K.H. Fritzemeier, U. Fuhrmann, B. Buchmann, T. Adevai, M.J. Murphy, M.B. Zelinski, B. Lindenthal, J.D. Hennebold, R.L. Stouffer, A prostaglandin E2 receptor antagonist prevents pregnancies during a preclinical contraceptive trial with female macaques, Hum. Reprod. 29 (2014) 1400–1412.

[25] Z. Liu, H.Y. Fan, Y. Wang, J.S. Richards, Targeted disruption of Mapk14 (p38MAPKalpha) in granulosa cells and cumulus cells causes cell-specific changes in gene expression profiles that rescue COC expansion and maintain fertility, Mol. Endocrinol. 24 (2010) 1794–1804.

[26] I. Ben-Ami, S. Freimann, L. Armon, A. Dantes, D. Strassburger, S. Friedler, A. Raziel, R. Seger, R. Ron-El, A. Amsterdam, PGE2 up-regulates EGF-like growth factor biosynthesis in human granulosa cells: new insights into the coordination between PGE2 and LH in ovulation, Mol. Hum. Reprod. 12 (2006) 593–599.

[27] S. Tamba, R. Yodoi, E. Segi-Nishida, A. Ichikawa, S. Narumiya, Y. Sugimoto, Timely interaction between prostaglandin and chemokine signaling is a prerequisite for successful fertilization, Proc. Natl. Acad. Sci. U. S. A. 105 (2008) 14539–14544.

[28] S.M. Harris, L.C. Aschenbach, S.M. Skinner, B.L. Dozier, D.M. Duffy, Prostaglandin E2 receptors are differentially expressed in subpopulations of granulosa cells from primate periovulatory follicles, Biol. Reprod. 85 (2011) 916–923.

[29] B.C. Vanderhyden, P.J. Caron, R. Buccione, J.J. Eppig, Developmental pattern of the secretion of cumulus expansion-enabling factor by mouse oocytes and the role of oocytes in promoting granulosa cell differentiation, Dev. Biol. 140 (1990) 307–317.

[30] K. Zamostna, J. Nevoral, R. Prochazka, M. Orsak, M. Sulc, V. Pajkosova, V. Pavlik, T. Zalmanova, K. Hoskova, T. Kott, F. Jilek, P. Klein, A simple method for assessment of hyaluronic acid production by cumulus-oocyte complexes, Czech. J. Anim. Sci. 61 (2016) 251–261.

[31] F. Nuttinck, P. Reinaud, H. Tricoire, C. Vigneron, N. Peynot, J.P. Mialot, P. Mermillod, G. Charpigny, Cyclooxygenase-2 is expressed by cumulus cells during oocyte maturation in cattle, Mol. Reprod. Dev. 61 (2002) 93–101.

[32] J. Liu, P.D. Carrière, M. Doré, J. Sirois, Prostaglandin G/H synthase-2 is expressed in bovine preovulatory follicles after the endogenous surge of luteinizing hormone, Biol. Reprod. 57 (1997) 1524–1531.

[33] A. Salustri, S. Ulisse, M. Yanagishita, V.C. Hascall, Hyaluronic acid synthesis by mural granulosa cells and cumulus cells in vitro is selectively stimulated by a factor produced by oocytes and by transforming growth factor-beta, J. Biol. Chem. 265 (1990) 19517–19523.

[34] Y. Yamashita, M. Okamoto, I. Kawashima, T. Okazaki, R. Nishimura, Y. Gunji, M. Hishinuma, M. Shimada, Positive feedback loop between prostaglandin E2 and EGF-like factors is essential for sustainable activation of MAPK3/1 in cumulus cells during in vitro maturation of porcine cumulus oocyte complexes, Biol. Reprod. 85 (2011) 1073–1082.

[35] U. Förstermann, B. Neufang, Elimination from the circulation of cats of 6-keto-prostaglandin E1 compared with prostaglandins E2 and I2, J. Pharm. Pharmacol. 35 (1983) 724–728.

[36] D.M. Duffy, Prostaglandin dehydrogenase (PGDH) in granulosa cells of primate periovulatory follicles is regulated by the ovulatory gonadotropin surge via multiple G proteins, Mol. Cell. Endocrinol. 333 (2011) 119–126.

[37] R. Prochazka, P. Kalab, E. Nagyova, Epidermal growth factor-receptor tyrosine kinase activity regulates expansion of porcine oocyte-cumulus cell complexes in vitro, Biol. Reprod. 68 (2003) 797–803.

[38] C.M. Wayne, H.Y. Fan, X. Cheng, J.S. Richards, Follicle-stimulating hormone induces multiple signaling cascades: evidence that activation of Rous sarcoma oncogene, RAS, and the epidermal growth factor receptor are critical for granulosa cell differentiation, Mol. Endocrinol. 21 (2007) 1940–1957.

[39] H.Y. Fan, Z. Liu, P.F. Johnson, J.S. Richards, CCAAT/enhancer-binding proteins (C/EBP)-α and -β are essential for ovulation, luteinization, and the expression of key target genes, Mol. Endocrinol. 25 (2011) 253–268.

[40] S.A. Ochsner, D.L. Russell, A.J. Day, R.M. Breyer, J.S. Richards, Decreased expression of tumor necrosis factor-alpha-stimulated gene 6 in cumulus cells of the cyclooxygenase-2 and EP2 null mice, Endocrinology 144 (2003) 1008–1019.

[41] N. Kimura, Y. Konno, K. Miyoshi, H. Matsumoto, E. Sato, Expression of hyaluronan synthases and CD44 messenger RNAs in porcine cumulus-oocyte complexes during in vitro maturation, Biol. Reprod. 66 (2002) 707–717.

[42] R. Prochazka, L. Nemcova, E. Nagyova, S. Scsukova, A. Mlynarcikova, Development of functional LH Receptors on pig cumulus-oocyte complexes cultured in vitro by a novel two-step culture system, Mol. Reprod. Dev. 76 (2009) 751–761.

[43] I. Kawashima, T. Okazaki, N. Noma, M. Nishibori, Y. Yamashita, M. Shimada, Sequential exposure of porcine cumulus cells to FSH and/or LH is critical for appropriate expression of steroidogenic and ovulation-related genes that impact oocyte maturation in vivo and in vitro, Reproduction 136 (2008) 9–21.

[44] R. Appeltant, J. Beek, L. Vandenberghe, D. Maes, A. Van Soom, Increasing the cAMP concentration during in vitro maturation of pig oocytes improves cumulus maturation and subsequent fertilization in vitro, Theriogenology 83 (2015) 344–352.