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Vliv gasotransmiterů na kumulární expanzi během meiotického zrání oocytů prasete

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doktorská disertační práce

Autor: **Ing. Jan NEVORAL**

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

Konzultant: **Ing. Mgr. Tereza Krejčová, Ph.D., KVD ČZU**

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PROHLÁŠENÍ

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podpis autora

SEZNAM ZKRATEK

AITI	Anaphase I/Telophase I
Akt	Protein Kinase B
APC/C	Anaphase Promoting Complex/Cyclosome
CaMKII	Calmodulin-Dependent Kinase II
cAMP	Cyclic Adenosine 3',5'-Monophosphate
CBS	Cystathionine β-Synthase
Cdc2	Cyclin-Dependent Kinase 2
Cdc25	Cyclin-Dependent Kinase 25
cGMP	Cyclic Guanosine 3',5'-Monophosphate
CEEFs	Cumulus Expansion Enabling Factors
CO	Carbon Monoxide
COCs	Cumulus-Oocyte Complexes
COX2	Cyclooxygenase 2
CSE	Cystathionine γ-Lyase
CSF	Cytostatic Factor
DOs	Denuded Oocytes
GAGs	Glycosaminoglycans
GC	Guanylate Cyclase
GDF9	Growth Differention Factor 9
GV	Germinal Vesicle
GVBD	Germinal Vesicle Breakdown
EGF	Epidermal Growth Factor
ELISA	Enzyme-linked Immunosorbent Assay
FITC	Fluorescein Isothiocyanate
FSH	Follicle Stimulating Hormone
H1	Histone H1
HA	Hyaluronic Acid
HABPs	Hyaluronic Acid Binding Proteins
HAS2	Hyaluronan-Synthase 2
HC	Heavy Chain (of Inter-α-trypsin Inhibitor)
HO	Heme Oxygenase
HPLC	High-Performance Liquid Chromatography
H_2S	Hydrogen Sulfide

HSP32	Heat Shock Protein 32
ChS	Chondroitin Sulfate
IGF-I	Insulin-like Growth Factor I
IαI	Inter-α-trypsin Inhibitor
IL-6	Interleukin-6
IP3K	Phosphoinositide-3-Kinase
IVF	*In Vitro* Fertilization
JNK	c-Jun N-terminal Kinase
LD	Late Diakinesis
LH	Luteinising Hormone
3-MPST	3-Merkaptopyruvate Sulfurtransferase
MI/II	Metaphase I/II
MAPK	Mitogen Activated Protein Kinases
MBP	Myeline Basic Protein
MPF	M-Phase/Maturation Promoting Factor
MTOC	Microtubule-Organizing Center
NO	Nitric Oxide
NOS	Nitric Oxide Synthase
OOXs	Oocytectomized Oocytes
PARP1	Poly(ADP-Ribose) Polymerase 1
PG2	Prostaglandin E2
PGs	Primordial Germ Cells
PKA	cAMP-Dependent Protein Kinase
PKC	Ca^{2+}-Dependent Protein Kinase
PKG	cGMP-Dependent Protein Kinase
Plk1	Polo-Like Kinase 1
RyR	Ryanodine Receptor
ROS	Reactive Oxygen Species
SCNT	Somatic Cell Nuclear Transfer
SDS	Sodium Dodecyl Sulphate
TGFβ	Transforming Growth Factor β superfamily
TNFAIP6	Tumour Necrosis Factor α-induced Protein 6

OBSAH

1. Úvod

Od úspěchu reprodukčních biotechnologií se odvíjí genetický pokrok ve šlechtění hospodářských zvířat i studium oogeneze a embryogeneze s možností využití v humánní medicíně. Vhodným modelem pro studium a následné využití poznatků v postupech asistované reprodukce je prase, jehož oocyty jsou využívány pro zrání v podmínkách *in vitro*. Zralé oocyty jsou následně používány pro *in vitro* oplození (IVF) a produkci embryí. Úspěšnost *in vitro* kultivace embryí je determinována vývojovou kompetencí a kvalitou meiotického zrání, během které oocyt získává schopnost aktivace a časného embryonálního vývoje.

Meiotické zrání oocytů je regulováno řadou faktorů, mezi které patří protein kinázy, molekuly druhých poslů, iontů a endogenně produkovaných plynů. V současné době je intenzivně studovaná skupina tzv. gasotransmiterů, plynných molekul s fyziologickým účinkem a potenciálem regulovat meiotické zrání oocytu. Skupina gasotransmiterů zahrnuje NO, H_2S a CO, jejichž tvorba je v buňkách katalyzována enzymy. Bylo zjištěno, že gasotransmiter NO sehrává úlohu v procesu meiotického zrání, aktivace oocytů a časného embryonálního vývoje. Zapojení gasotransmiteru NO lze předpokládat rovněž v procesu označovaném jako stárnutí oocytu, během kterého dochází ke snižování kvality *in vitro* dozrálých oocytů.

Současně s meiotickým zráním dochází ke kumulární expanzi kumulo-oocytárního komplexu. Kumulární expanze spočívá v syntéze glykosaminoglykanů, zejména hyaluronové kyseliny, do extracelulárního prostoru kumulárních buněk. Syntéza hyaluronové kyseliny a intenzita kumulární expanze je determinována velikostí folikulů, kvalitou obalu kumulárních buněk a biochemickými změnami během meiotického zrání oocytu. Proto je možné využít kumulární expanzi jako hodnotící marker kvality oocytu. Kumulární expanze a meiotické zrání jsou regulovány signálními drahami, zahrnující gonadotropní hormony, růstové faktory či klíčové protein kinázy. Do řízení kumulární expanze a zrání oocytů jsou zapojeny také molekuly gasotransmiterů, jak bylo popsáno na efektu NO. Úloha gasotransmitrů H_2S a CO během reprodukčních procesů včetně meiotického zrání oocytů a kumulární expanze zůstává neobjasněna a jejich poznání je užitečné pro efektivní kultivaci a úspěšné využití oocytů v podmínkách *in vitro*.

2. Literární přehled

2.1. Oogeneze savců

2.1.1. Oogeneze, folikulogeneze a růst oocytu

Gamety savců vznikají z primordiálních zárodečných buněk (PGCs - primordial germ cells) již během prenatálního vývoje. V epitelu embryonálních vaječníků podléhají PGCs intenzivnímu mitotickému dělení a diferenciaci, čímž dávají vzniknout vysokému počtu oogonií, které se po několik následujících dní embryonálního vývoje dále mitoticky dělí (Wassarman, 1988). Omezené množství PGCs přetrvává ve tkáni vaječníku do dospělosti samice, kdy slouží jako zdroj oogonií i v adultní fázi ontogeneze (Johnson *et al*., 2004). Oogonie vstupují do meiotického dělení, kde je vývoj následně zablokován v diktyotene profáze 1. meiotického dělení. Takové buňky jsou označovány jako diktyotenní oocyty v 1. meiotickém bloku (Wassarman, 1988).

Již v prenatální fázi ontogeneze dochází k separaci jednotlivých oogonií a jejich obalení pre-graulózními buňkami, čímž vznikají tzv. primordiální folikuly. Oogonie v těchto folikulech zahajují meiotické dělení a pre-granulózní buňky se začínají mitoticky dělit a diferencovat v granulózní buňky. Z vrstvy granulózních buněk obklopující bezprostředně oocyt se v pokročilých stádiích folikulogenze diferencují tzv. kumulární buňky včetně *corona radiata*, které zprostředkovávají spojení s oocytem, prostřednictvím výběžků prostupujících *zonu pellucidu* a buněčného spojení typu gap junction (Brower *et* Schultz, 1982; Wassarman, 1988).

Další průběh oogeneze probíhá ve folikulu současně s procesem folikulogenze, který u pohlavně dospělých prasnic trvá více než 80 dní (Morbeck *et al*., 1992). Jen folikuly s průměrem větším než 3 mm obsahují oocyty, které jsou schopné úspěšně dokončit meiotické zrání (Marchal *et al*., 2002). Tyto oocyty prodělávají během folikulogeneze proces růstu, který determinuje tzv. meiotickou kompetenci oocytu.

Do fáze růstu vstupují oocyty v 1. meiotickém bloku. Během fáze růstu dochází k přibývání buněčných organel, které současně mění svoji ultrastrukturu. Jádro oocytu se zvětšuje a jadérko nabývá kompaktnější podoby. Během růstu dochází k intenzivní transkripci a tvorbě RNA, kdy roste počet ribozómů, polyzómů i množství proteinů, které jsou v polyzómech syntetizované. Se zvyšující se intenzitou proteosyntézy dochází k zvětšování endoplazmatického retikula a Golgiho komplexu (Wassarman, 1988). Proteosyntéza a post-translační modifikace kumulujících se proteinů v rostoucím oocytu je nezbytná pro následné meiotické zrání. Mezi tyto proteiny patří strukturální proteiny, jako jsou histony, tubulin a

glykoproteiny *zony pellucidy*, a enzymy klíčové pro meiotické zrání. Enzymy jsou během zrání kumulovány a udržovány v inaktivní formě pro-enzymů. Dostatečné množství klíčových faktorů meiotického zrání determinuje meiotickou kompetenci, kterou disponují plně dorostlé oocyty, které u prasat dosahují velikosti 120 – 125 μm (Schultz *et al*., 1979; Wassarman, 1988; Yanagimachi, 1988; Wassarman *et* Albertini, 1994).

2.1.2. Meiotické zrání oocytů

Znovu zahájit a plně dokončit meiotické zrání jsou schopny pouze diktyotenní oocyty plně dorostlé ve stádiu zárodečného váčku (GV - germinal vesicle). Uvolnění 1. meiotického bloku a znovu zahájení meiotického zrání je charakteristické procesem označovaným jako rozpad zárodečného váčku (GVBD - germinal vesicle breakdown). Po GVBD vstupuje oocyt do metafáze 1. meiotického dělení, metafáze I. Následně dochází k segregaci chromozómů, dosažení anafáze I, telofáze I a vydělení 1. pólocytu (Motlík *et* Fulka, 1976). Po dosažení metafáze II je meiotické dělení oocytu znovu spontánně přerušeno (Yanagimachi, 1988).

GVBD a znovu zahájení meiotického dělení nastává v *in vivo* podmínkách v důsledku hormonální stimulace působením gonadotropinů - folikuly stimulujícího hormonu (FSH) a luteinizačního hormonu (LH). Hormonální stimulace má za následek potlačení inhibičních faktorů jako je cAMP a cAMP-dependentní kináza (PKA) v kumulárních buňkách a oocytech (Eppig, 1991). V podmínkách *in vitro* je možné využití FSH a LH v kombinaci se sérovými proteiny a růstovými faktory (Singh *et al*., 1997; Uhm *et al*., 1998).

Signální dráha cAMP/PKA v kumulárních buňkách a oocytech je odpovědná za udržení 1. meiotického bloku v oocytech (Eppig, 1991). Syntéza cAMP je katalyzována adenylát-cyklázou (Eppig, 1989), která je lokalizovaná v cytoplazmatické membráně oocytu (Liang *et al*., 2005). Pokles koncentrace cAMP je důsledkem potlačení aktivity adenylát-cyklázy a aktivace fosfodiesteráz štěpících dvojnou vazbu cAMP (Dekel *et* Beers, 1980). Podobně jako cAMP, se na regulaci meiotického zrání podílí molekula jiného buněčného posla, cGMP (Tornell *et al*., 1984, 1990). Snížení množství cGMP v kumulárních buňkách umožňuje aktivaci fosfodiesteráz v oocytu a tak pokles koncentrace cAMP v oocytu (Norris *et al*., 2009). Koncentrace cAMP v oocytu klesá rovněž v důsledku přerušení mezibuněčných spojů *gap junction* mezi oocytem a kumulárními buňkami po stimulaci zrání oocytu a omezení toku cAMP do oocytu z kumulárních buněk (Liang *et al*., 2007). Pokles koncentrace cAMP a aktivity PKA je nezbytný pro GVBD (Dekel *et* Beers, 1980; Dekel *et al*., 1981; Schultz *et al*., 1983; Wassarman, 1988). V průběhu meiotického zrání přetrvává nízká koncentrace cAMP v oocytu (Dekel *et al*, 1988).

Současně s poklesem koncentrace inhibičních faktorů dochází během meiotického zrání k uvolnění Ca^{2+} iontů z endoplazmatického retikula do cytoplazmy prostřednictvím inositol-trifosfátových (IP3R) a ryanodinových receptorů (RyR) (Caroll *et al*., 1994; Macháty *et al*., 1997). Ca^{2+} následně aktivuje protein-kinázy, jako je kalmodulin-dependentní kináza (CaMKII) (Fan *et al*., 2003) a některé izoformy Ca^{2+}-dependentní protein-kinázy (PKC) (Fan *et al*., 2002a; Fan *et* Sun, 2004). Tyto kinázy se společně s cyklin-dependentní kinázou 25 (Cdc25) (Taieb *et al*., 1997), polo-like kinázou 1 (Plk1) (Pahlavan *et al*., 2000) a protein-kinázou B (Akt) (Cecconi *et al*., 2010) uplatňují v regulaci klíčových faktorů meiotické zrání – M-fázi/zrání podporujícího faktoru (MPF) a mitogeny aktivované protein kinázy (MAPK) (Motlík *et* Kubelka, 1990; Sobajima *et al*., 1993).

MPF nabývá podoby heterodimeru, který v oocytech sestává z katalytické podjednotky Cdc2 a regulační podjednotky cyklinu B. Cdc2 v GV-oocytu je inhibičně fosforylována na threoninu-14 a tyrozinu-15 domény p34. Takto fosforylovaný heterodimer Cdc2-cyklin B je inaktivní a je označován jako pre-MPF. Dostatečné množství pre-MPF, syntetizovaného během fáze růstu, je nezbytné pro úspěšné meiotické zrání (Norbury *et* Nurse, 1992; Christmann *et al*., 1994). Za inhibiční fosforylaci MPF jsou odpovědné kinázy Wee1 a Myt1 (Taieb *et al*., 1997).

Pro aktivaci MPF je nutná defosforylace threoninu-14 a tyrozinu-15 pomocí Cdc25 (Taieb *et al*., 1997). Na aktivaci MPF v prasečích oocytech se významně podílí také Plk1 (Anger *et al*., 2004), která potlačuje Myt1 (Okano-Uchida *et al*., 2003) a současně aktivuje Cdc25 (Kumagai *et* Dunphy, 1996). Během aktivace MPF dále dochází k fosforylaci cyklinu B na serinových zbytcích Ser-94 a Ser-96 (Izumi *et* Maller, 1991). Takto aktivovaná MPF katalyzuje další defosforylaci pre-MPF prostřednictvím další aktivace Cdc25. Tato autoakcelerační smyčka má za následek prudký vzestup aktivity MPF (Lee *et al*., 1999; Kikuchi *et al*., 2000; Fan *et al*., 2002b; Kishimoto, 2003). Aktivní MPF je dále odpovědný za fosforylaci histonů a kondenzaci chromozómů (Arion *et al*., 1988), za fosforylaci jaderných laminů a jejich depolymeraci (Lüscher *et al*., 1991), čímž v oocytech indukuje GVBD (Motlík *et al*., 1998a). Mimoto se MPF podílí na regulaci MAPK signální kaskády (Fan *et al*., 2002b). Aktivita MPF klesá se vstupem oocytu do anafáze I a telofáze I (Verlhac *et al*., 1993, 1994). Pokles MPF aktivity je nutný pro přechod z meiózy I do meiózy II, segregaci chromozómů a vydělení 1. pólocytu (Hampl *et* Eppig, 1995). Pokles aktivity MPF spočívá v degradaci katalytické podjednotky MPF - cyklinu B, pomocí polyubiquitinace anafázi-podporujícím komplexem/cyklozómem (APC/C) a následné proteolýzy v proteasomu S26 (Peters, 2002; Yi

et al., 2008). Aktivita MPF následně opět vzrůstá a dosahuje maxima v metafázi II, kde se podílí na udržení 2. meiotického bloku (Naito *et al*., 1995; Taieb *et al*., 1997).

Další významnou skupinou faktorů regulujících meiotické zrání je rodina Ser/Thr kináz, MAPK. V savčích oocytech hrají významnou roli izoformy MAPK1 a 2 (Inoue *et al*., 1998; Fan *et al*., 2002b; Ohashi et al., 2003; Tao *et al*., 2005b). V aktivaci MAPK se uplatňuje transdukce signálu prostřednictvím proteinů Mos a MEK (Kiriakis *et* Avruch, 2001; Roux *et* Blenis, 2004). Do regulace signální dráhy Mos/MEK/MAPK je v procesu meiotického zrání prasečích oocytů zapojen gonadotropin FSH (Li *et al*., 2002), cAMP (Liang *et al*., 2005) a MPF (Fan *et al*., 2002b). Aktivita MAPK v prasečích oocytech vzrůstá krátce před GVBD iniciované MPF (Wehrend *et* Meinecke, 2001). Na rozdíl od MPF, během meiotického zrání přetrvává MAPK aktivní (Lee *et al*., 2000; Villa-Diaz *et* Miyano, 2004). Důležitým substrátem aktivní MAPK je ribozóm S6-kináza $p90^{rsk}$ (Fan *et al*., 2002b; Kishimoto, 2003; Fan *et* Sun, 2004; Roux *et* Blenis, 2004), prostřednictvím které MAPK participuje na opětovné syntéze cyklinu B v průběhu meiotického zrání a na opakované aktivaci MPF při přechodu meiózy I a II (Gross *et al*., 2000; Ohashi *et al*., 2003). MAPK současně potlačuje aktivitu Myt1 prostřednictvím Plk1, čímž zabraňuje inhibiční fosforylaci na Cdk2 a konverzi do pre-MPF (Fan *et al*., 2002b; Kishimoto, 2003; Ohashi *et al*., 2003), a udržuje aktivní Cdc25 (Kishimoto, 2003). Substráty MAPK jsou také další regulační faktory meiotického zrání, jako je PKA, PKC a CaMKII (Kishimoto, 2003; Fan *et* Sun, 2004).

MAPK se přímo podílí na GVBD prostřednictvím aktivace MPF (Ohashi *et al*., 2003), přestože není pro GVBD prasečích oocytů nezbytná (Fan *et al*., 2002b). Aktivní MAPK je důležitá pro kondenzaci chromozómů a jejich segregaci během meiotického dělení (Kishimoto, 2003). Současně se podílí na fosforylaci mikrotubuly organizujícího centra (MTOC), na formaci dělícího vřeténka, jeho stabilizaci a elongaci, důležitých pro vydělení 1. pólocytu (Inoue *et al*., 1995, 1998; Lee *et al*., 2000; Li *et al*., 2002; Takakura *et al*., 2005). Mimoto, MAPK potlačuje kompletaci jaderné membrány fosforylací jaderných laminů (Inoue *et al*., 1998; Fan *et al*., 2002b). Aktivní MAPK způsobuje migraci dělícího vřeténka k membráně oocytu, asymetrickou cytokinezi a vydělení 1. pólocytu (Tong *et al*., 2003). V důsledku aktivní MAPK tak nenastává interfáze v přechodu meiózy I a II, nedochází k replikaci DNA a zahájení mitotického dělení ihned po meióze I (Fan *et* Sun, 2004; Sasaki *et* Chiba, 2004; Kishimoto, 2003). Aktivita MAPK přetrvává v savčích oocytech včetně prasete až do dosažení metafáze II, kde se podílí na udržování vysoké aktivity cytostatického faktoru (CSF) a 2. meiotického bloku (Maller *et al*., 2001; Ohashi *et al*., 2003; Ito *et al*., 2004).

Meiotické zrání oocytů je spontánně zablokováno v metafázi II (Yanagimachi, 1988). Za udržování 2. meiotického bloku je odpovědný CSF (Masui *et* Markert, 1971), jehož klíčovými komponentami jsou Emi1 a Mos (Li *et al*., 2002; Reimann *et* Jackson, 2002). Aktivita CSF vzrůstá po dokončení meiózy I a přetrvává do metafáze II (Takakura *et al*., 2005). Úloha CSF spočívá v potlačení aktivity APC/C a proteolytické degradace cyklinu B (Maller *et al*., 2001; Reimann et Jackson, 2002). Výsledkem je stabilizace MPF a udržování jeho konstantní aktivity (Maller *et al*., 2002; Fan *et* Sun, 2004). Aktivní MPF současně fosforyluje protein Emi1 a tím pozitivní zpětnou vazbou udržuje vysokou aktivitu CSF (Kishimoto, 2003). Mos, druhá z komponent CSF, se uplatňuje v udržování aktivity Mek a MAPK (Kyriakis *et* Avruch, 2001).

2.1.3. Aktivace a stárnutí oocytů

Dozrálé MII-oocyty jsou předurčené k oplození spermií, aktivaci a embryonálnímu vývoji (Wassarman, 1988; Yanagimachi, 1988). Spermie přináší do oocytu cytoplazmatický faktor fosfolipázu Cζ, která produkuje signální molekuly IP3. Po navázání na receptory IP3R, lokalizované na endoplazmatickém retikulu oocytu, dochází k vyplavení intracelulárního Ca^{2+} do cytoplazmy oocytu (Macháty *et al*., 1997; Yoo *et* Smith, 2007). To má za následek aktivaci CaMKII (Fan *et al*., 2002b; Fan *et* Sun, 2004) a následné snížení aktivity CSF. To je spojeno rovněž s poklesem aktivity MPF a MAPK (Kikuchi, 2000). Biochemické změny vedou k ztvrdnutí *zony pellucidy* a zvětšení perivitelinního prostoru jako prevence proti polyspermii (DeMeestere *et al*., 1997; Ueno *et al*., 2009).

Pokud nedochází k oplození oocytu, nastává spontánně jev, který je komplexně označován jako stárnutí oocytu (Petrová *et al*., 2004, 2005). Stárnutí zahrnuje spontánní pokles faktorů odpovědných za udržování 2. meiotického bloku, uvolnění tohoto bloku a v důsledku spontánní partenogenetickou aktivaci oocytu (Lindemann *et* Goltz, 1986). V případě delšího trvání meiotického bloku dochází k aktivaci apoptotických signálních drah, zahrnující c-Jun N-terminální kinázu (JNK) a pro-apoptotické faktory z rodiny Bcl-2. Tyto změny vedou k programované buněčné smrti (Lei *et al*., 2002; Petrová *et al*., 2009). Tyto procesy lze v *in vitro* podmínkách navodit tzv. prodlouženou kultivací.

2.2. Kumulární expanze

Současně s meiotickým zráním oocytu v podmínkách *in vivo* a *in vitro* syntetizují kumulární buňky strukturálních komponenty extracelulární matrix, čímž dochází ke zvětšování COC. Proces přibývání a formování mezibuněčné hmoty v kumulárním obalu během meiotického

zrání se označuje jako kumulární expanze (Dekel *et al*., 1979; Eppig, 1979). V *in vivo* podmínkách dochází ke kumulární expanzi uvnitř folikulu bezprostředně před ovulací. Kumulární expanze probíhá rovněž během kultivace COCs v *in vitro* podmínkách.

Komponenty extracelulární hmoty představují komplexy proteoglykanů: glykosaminoglykanů (GAGs) a membránových proteinů kumulárních buněk (Moscatelli *et* Rubin, 1974). Nejčetněji zastoupeným GAG v kumulu je hyaluronová kyselina (HA), méně chondroitin sulfát, keratansulfát a heparansulfát (Tirone *et al*., 1993; Nakayama *et al*., 1996).

Endokrinní regulace kumulární expanze spočívá v působení gonadotropních hormonů luteinizační hormon (LH) a folikuly stimulující hormon (FSH) (Dekel *et al*., 1979). Gonadotropní hormony ovlivňují metabolismus granulózních buněk (Wassarman, 1988), které do folikulární tekutiny produkují růstové faktory, jako je epidermální růstový faktor (EGF) a inzulínu podobný růstový faktor (IGF-I) (Daen *et al*., 1994; Nakayama *et al*., 1996; Motlík *et al*., 1998b; Ježová *et al*., 2001; Němcová *et al*., 2007; Zhang *et al*., 2008). Růstové faktory regulují kumulární expanzi prostřednictvím protein-ináz MAPK, PI3K a Akt (Němcová *et al*., 2007; Procházka *et al*., 2012).

Folikulární tekutina současně obsahuje množství inhibičních faktorů meiotického zrání a kumulární expanze, jako cAMP a PKA, které jsou odpovědné za inhibiční efekt ve folikulech plně nedorostlých (Qian *et al*., 2003; Yang *et al*., 1993; Nandi *et al*., 2007). V rostoucím folikulu dochází ke snižování koncentrace inhibujících látek a současně k hromadění steroidních hormonů a stimulujících faktorů parakrinní regulace meiotického zrání (Yoshida *et al*., 1992; Ding *et* Foxcroft, 1994; Dode *et* Graves, 2002).

Na formování kumulární expanze se významně podílí proteolýza IαI pomocí ubiquitin-proteasomálního systému (Yi *et al*., 2008). Štěpení IαI na těžké řetězce (HC), HC1 a HC2, je nezbytná pro vazbu na HA a stabilizuje extracelulární matrix, čímž se proteasom stává pro kumulární expanzi prasečích COCs nezbytným (Nagyová *et al*., 2004, 2012).

Kumulární expanzi přímo stimuluje plně dorostlý oocyt, který v průběhu meiotického zrání produkuje faktory podporující kumulární expanzi (CEEFs) (Salustri *et al*., 1989; Eppig *et al*., 1993; Tirone *et al*., 1993). Ve skupině CEEFs byly později identifikovány mnohé růstové faktory zejména z rodiny transformujících růstových faktorů β (TGFβ), zahrnující TGFβ1, TFGβ2 a růstový diferenciační faktor 9 (GDF9), (Vanderhyden *et al*., 2003; Dragovic *et al*., 2005). Během meiotického zrání, zejména po dosažení meatafáze I, zjevně ustává sekreční aktivita oocytu v produkci faktorů stimulujících kumulární expanzi (Nagyová *et al*., 2000).

Faktory, které regulující kumulární expanzi prostřednictvím těchto a dalších signálních drah, mohou být molekuly gasotransmiterů. Bylo zjištěno, že NO-syntáza a přítomnost NO je nutná

pro úplnou kumulární expanzi oocytů (Tao *et al*., 2005b; Amale *et al*., 2011). Přesnější mechanizmus NO v procesu kumulární expanze prozatím není objasněn.

Kumulární buňky jsou svojí metabolickou aktivitou důležité pro buněčnou signalizaci, nabytí meiotické a vývojové kompetence oocytu během růstu oocytu (Fukui, 1990; Qian *et al*., 2003; Han *et al*., 2006; Auclair *et al*., 2013). Počet vrstev kumulárních buněk a vstupní kvalita kumulu následně rozhoduje o úspěšnosti meiotického zrání (Procházka *et al*., 2000; Karja, 2008). Od charakteru kumulu se odvíjí intenzita kumulární expanze, která pozitivně koreluje s úspěšností meiotického zrání *in vitro* (Qian *et al*., 2003). Úplné odstranění kumulu z oocytu tak má za následek ztrátu schopnosti dokončit meiotické zrání *in vitro*, u dozrálých oocytů způsobuje potlačení oplození schopnosti a ztrátu vývojové kompetence oocytu (Han *et al*., 2006; Auclair *et al*., 2013). Přítomnost kumulárních buněk a jejich expanze jsou tak nezbytné pro vydělení 1. pólocytu během meiotického zrání a příznivě ovlivňuje přežitelnost zrajících oocytů (Ju *et* Rui, 2012). Ze zmíněných důvodu se pro *in vitro* zrání používají jen oocyty s neporušeným obalem kumulárních buněk v několika vrstvách a kumulární expanze je následně využívána jako hodnotící marker pro kvalitu meiotického zrání oocytu a nabytí vývojové kompetence.

Kumulární buňky mezi sebou a oocytem navzájem komunikují prostřednictvím molekul druhých poslů, zejména cAMP, cGMP a Ca^{2+} iontů, procházejících do oocytu buněčnými spoji gap junction (Moor *et al*., 1980; Yanagimachi, 1988). Molekuly druhých poslů v oocytu regulují signální dráhy protein-kináz, jako je PKA, PKC a zprostředkovaně také MPF a MAPK (Tatemoto *et* Terada, 1998; Su *et al*., 2003).

Kumulární expanze je spojená s rostoucí vzdáleností mezi kumulárními buňkami v důsledku syntézy HA, morfologickými změnami kumulárních buněk, endocytózou proteinů buněčných spojů a přerušením toku inhibičních látek mezi kumulárními buňkami a oocytem během jeho meiotického zrání (Chen *et al*., 1990; Šutovský *et al*., 1994; Prydz *et* Dalen, 2000).

Mimo mechanické funkce plní HA v COC také úlohu signální molekuly - ligandu. Receptorem HA jsou molekuly ze skupiny proteinů schopné vázat hyaluronovou kyselinu (HABPs) a podílet se na tvorbě glykoproteinů. Do skupiny HABP patří receptor CD44, který je lokalizován v cytoplazmatické membráně kumulárních buněk a cytoplazmě oocytu (Kimura *et al*., 2002; Yokoo *et al*., 2002). CD44 receptory oocytu se po navázání HA podílejí na regulaci meiotického zrání oocytu (Kimura *et al*., 2002; Yokoo *et al*. (2007), prostřednictvím fosforylace proteinů gap junction, uzavření tohoto buněčného spojení a přerušením toku cAMP z kumulárních buněk do oocytu (Yokoo *et al*., 2010). Efekt CD44 receptoru aktivovaného HA spočívá také v autoregulaci kumulární expanze a její amplifikaci

(Yokoo *et al*., 2003), přestože aktivace CD44 není pro kumulární expanzi nezbytná (Yokoo *et al*., 2007).

Ze skupiny HABP se v kumulární expanzi dále uplatňuje inhibitor inter-α-trypsinu (IαI) a protein 6 indukovaný faktorem nádorové nekrózy α (TNFAIP6) (Chen *et al*., 1992; Fülöp *et al*., 1997). Během kumulární expanze jsou tyto proteiny odpovědné za formování expandovaného kumulu stabilizací řetězců HA (Nagyová *et al*., 2004, 2012).

2.3. Úloha gasotransmiterů ve fyziologii reprodukce

Mezi možné regulační faktory meiotického zrání, aktivace oocytů a jejich stárnutí patří skupina jednoduchých plynných molekul s fyziologickým účinkem označovaná jako gasotransmitery. Molekuly gasotransmiterů jsou ve fyziologických koncentracích enzymaticky uvolňovány v živočišných buňkách, ve kterých plní úlohu druhých poslů a jsou tak zapojeny do signální komunikace (Mustafa *et al*., 2009b). Významnou úlohu sehrávají gasotransmitery v reprodukčních procesech (shrnuto v Šmelcová *et* Tichovská, 2011).

Do skupiny gasotransmiterů patří oxid dusnatý (NO), oxid uhelnatý (CO) a sulfan (H_2S). V živočišných buňkách dochází k interakcím gasotransmiterů a k vzájemnému propojení jejich signálních drah (shrnuto v Olson *et al*., 2012).

Fyziologická produkce NO je zprostředkována NO-syntázou (NOS), která katalyzuje konverzi L-argininu na citrulin a NO (Kwon *et al*., 1990; Lamas *et al*., 1992). NOS existuje ve třech izoformách kódovaných samostatnými geny: endoteliální NOS (eNOS), neuronová NOS (nNOS) a indukovatelná NOS (iNOS). Zatímco jsou eNOS a nNOS Ca^{2+}-dependentní a produkují jen malé množství NO během krátké doby nanejvýše několika minut (Lamas *et al*., 1992; Bredt *et al*., 1991), činnost iNOS je nezávislá na Ca^{2+} iontech a produkuje stabilně 100 – 1000x více NO během několika hodin (Nathan, 1992; Xie *et al*., 1992). Přítomnost všech izoforem NOS byla detekována v oocytech a kumulárních buňkách prasete (Hattori *et al*., 2000; Chmelíková *et al*., 2010; Ding *et al*., 2012).

Produkce NO byla studována v reprodukčních procesech samců i samic. Molekula NO se uplatňuje v sekreci gonadotropních hormonů (McCann, 1982), čímž je zapojena do produkce testosteronu, regulace spermatogeneze a pohlavního chování (Davidoff *et al*., 1997). Signální kaskáda NO je rovněž zapojena do řízení erekce penisu (Burnett, 2002). NO ovlivňuje hormonální sekreci gonadotropinů a steroidních hormonů také v samičím reprodukčním systému (Van Voorhis *et al*., 1995), kde se podílí v řízení estrálního cyklu (Jablonka-Shariff

et al., 1999), folikulogeneze, ovulace a meiotického zrání oocytu (Jablonka-Shariff *et* Olson, 1998; 2000).

Bylo zjištěno, že molekula NO plní významnou úlohu v procesu meiotického zrání, kde je nezbytná pro přechod z meiózy I do meiózy II (Jablonka-Shariff *et* Olson, 1998). Genový knock-out myších samic nebo použití inhibitoru NOS během zrání myších oocytů *in vitro* má za následek vyšší podíl oocytů, které zůstaly v metafázi I anebo podlehly degenerativním změnám (Jablonka-Shariff *et* Olson, 2000). Podobný efekt má inhibice syntézy NO také v oocytech skotu (Schwartz *et al*., 2008) a prasete (Tao *et al*., 2005a). V prasečích oocytech kultivovaných v podmínkách *in vitro* je NOS a produkce NO nezbytná pro GVBD a dosažení metafáze 2. meiotického dělení. Je zřejmé, že na meiotickém zrání prasečích oocytů se významně podílí NOS syntetizovaná v kumulárních buňkách (Chmelíková *et al*., 2010). Inhibice NOS v ovčích COCs kultivovaných *in vitro* rovněž způsobuje potlačení kumulární expanze (Amale *et al*., 2011).

Úloha NO byla studována také v aktivaci oocytů a průběhu časného embryonálního vývoje inseminovaných a partenogeneticky aktivovaných oocytů (Goud *et al*., 2008; Krejčová *et al*., 2009). NO není nezbytný pro oplození myších oocytů (Hyslop et al., 2001), je však schopný aktivovat oocyty prasat a žab *Xenopus laevis* (Petr *et al*., 2005, 2010; Ješeta *et al*., 2012).

Molekula NO se uplatňuje v aktivaci membránového proteinu guanylát-cyklázy (GC) a produkci cGMP, který v buňkách funguje jako druhý posel (Bellamy *et al*., 2002). Signální kaskáda NO/GC/cGMP je nezbytná pro gametogenezi a časnou folikulogenezi prasečích ovarií již během embryonálního vývoje (Ding *et al*., 2012). Vysoká koncentrace cGMP je spojena s aktivitou cGMP-dependentní protein-kinázy (PKG) a meiotickým blokem oocytů (Zhang *et al*., 2005). Účinek molekuly NO v procesu meiotického zrání tak pravděpodobně nespočívá ve zvýšení koncentrace cGMP (Bilodeau-Goeseels, 2007). V procesu aktivace oocytu se však NO uplatňuje prostřednictvím regulace signální dráhy cGMP a PKG (Petr *et al*., 2006).

Možným mechanizmem účinku NO v reprodukčních procesech je nitrosylace proteinů (Iwakiri, 2011). NO ovlivňuje prostřednictvím nitrosylace např. aktivitu ryanodinových receptorů a uvolnění Ca^{2+} iontů z intracelulárních depozit (Xu *et al*., 1998). Uvolnění Ca^{2+} iontů po působení NO na ryanodinové receptory je možným mechanismem regulace meiotického zrání a aktivace oocytu.

H_2S je endogenně uvolňován z L-cysteinu pomocí enzymů cystathionine β-syntházy (CBS), cystathionine γ-lyázy (CSE) a 3-merkaptopyruvát sulfurtransferázy (3-MPST) (Wang, 2002;

Shibuya *et al*., 2009). Přítomnost enzymů uvolňujících H_2S byla detekována v granulózních buňkách folikulů a kumulárních buňkách myších COCs (Liang R. *et al*., 2006, 2007). CBS a CSE byly lokalizovány v buňkách dělohy a placenty potkanů (Patel *et al*., 2009). Dosud nebyla prokázána přítomnost 3-MPST v buňkách reprodukční soustavy.

Ve tkáni samčí reprodukční soustavy bylo prokázáno, že H_2S potlačuje relaxaci kavernózního tělesa pyje a sehrává úlohu v řízení erekce (Ghasemi *et al*., 2012). V samičí reprodukční soustavě bylo zjištěno, že exprese CBS v ovariu a kumulárních buňkách myší je nezbytná pro vývoj folikulů a pro zrání oocytů *in vitro* (Liang R. *et al*., 2007). Efekt inhibice enzymů uvolňujících H_2S je vysvětlován absencí H_2S anebo hromaděním L-cysteinu a tzv. hyperhomocysteinémií (Liang R. *et al*., 2007; Sen *et al*., 2010).

Endogenně uvolněný H_2S se ve vodném prostředí buňky rychle hydrolyzuje na ionty S^{2-} a HS^- (Shen *et al*., 2012). Možným mechanizmem účinku těchto iontů je konverze –SH skupiny cysteinu na -SSH skupinu procesem nazývaným S-sulfhydratace (Mustafa *et al*., 2009a). Efekt H_2S prostřednictvím S-sulfhydratace spočívá v post-translačních modifikacích proteinů (Sen *et al*., 2012), regulaci K_{ATP} kanálových proteinů (Kang *et al*., 2012) a zvýšení katalytické aktivity enzymů (Paul *et* Snyder, 2012). Bylo tak zjištěno, že H_2S je prostřednictvím regulace K_{ATP} kanálů zapojen do řízení iontové rovnováhy na buněčných membránách (Zhang *et al*., 2007). H_2S se způsobem S-sulfhydratace podílí také na regulaci klíčových faktorů M-fáze buněčného cyklu somatických buněk prostřednictvím signálních drah cAMP/PKA (Njie-Mbye *et al*., 2012) a PI3K/Akt (Huang *et al*., 2010; Ornelas *et al*., 2013). Lze předpokládat, že prostřednictvím těchto signálních drah sehrává H_2S úlohu také v regulaci meiotického zrání oocytů a kumulární expanze.

Produkci CO v buňkách katalyzuje hem-oxygenáza (HO), která se vyskytuje ve třech izoformách: HO1, HO2 a HO3 (Maines, 1988; McCoubrey *et al*., 1997). HO patří do skupiny proteinů tepelného šoku (HSPs) a je označována také jako HSP32 (Ewing *et al*., 1992). Substrátem HO je molekula hemu, která je degradována na CO a biliverdin (Tenhuen *et al*., 1968).

Endogenně produkovaná molekula CO plní v buňkách živočichů úlohu druhého posla (Marks *et al*., 1991). CO má efekt vasorelaxantu (Chen *et al*., 2003b) a regulátoru zánětlivých procesů (Freitas *et al*., 2006). Bylo rovněž zijištěno, že signální molekula CO v organizmu plní úlohu neurotransmiteru (Johnson *et* Johnson, 2000),

HO v reprodukčních orgánech byla detekována ve varlatech potkanů (Trakshel *et al*., 1986), děloze potkanů (Kreiser *et al*., 2003) a v granulózních buňkách prasečích folikulů (Harada

et al., 2004). Bylo zjištěno, že exprese HO je nezbytná pro úplnou funkci myších ovarií, růst folikulů a ovulaci oocytu (Zenclussen *et al*., 2012).

Jeden ze způsobů, jak CO reguluje reprodukční procesy, je řízení hormonální sekrece (Kostoglou-Athanassiou *et al*., 1996). Bylo zjištěno, že CO se uplatňuje v řízení steroidogeneze a sekrece progesteronu a estradiolu v ovariální tkáni (Alexandreanu *et* Lawson, 2003). Potlačená exprese HO genovým knock-outem u myší má za následek ovulaci omezeného počtu oocytů a snížení jejich schopnosti oplození (Zenclussen *et al*., 2012). Vliv CO byl prokázán také v průběhu gravidity, kdy CO potlačuje kontrakci hladkosvalových buněk dělohy, čímž reguluje nástup porodu (Bainbridge *et* Smith, 2005).

Jednou z cílových molekul CO je guanylát-cykláza, která je odpovědná za produkci buněčného posla cGMP (Morita *et al*, 1995). CO se tak podílí na regulaci koncentrace cGMP také ve tkáních reprodukčního systému, např. v hladkosvalových buňkách dělohy (Cella *et al*., 2006). Podobně jako v somatických buňkách, lze očekávat zapojení CO do regulace koncentrace cGMP v kumulárních buňkách a oocytu.

3. Hypotéza a cíle

Byla stanovena hypotéza, že gasotransmitery jsou zapojeny do regulace meiotického zrání oocytů, kumulární expanze kumulo-oocytárních komplexů (COCs) a stárnutí oocytů prasete kultivovaných v *in vitro* podmínkách.

Cílem práce bylo:

a) zhodnotit vliv donoru H_2S na průběh meiotické zrání oocytů;
b) zhodnotit vliv donoru H_2S na kumulární expanzi COCs;
c) zhodnotit vliv donoru H_2S na získávání vývojové kompetence oocytů během meiotického zrání;
d) zhodnotit průběh stárnutí oocytů na základě markerů časné apoptózy – fragmentace DNA a exprese pro-apoptotických faktorů, po inhibici c-Jun N-terminální kinázy;
e) popsat distribuci NO syntázy a zhodnotit vliv její inhibice ve stárnoucích oocytech.

4. Materiál a metodika

4.1. Izolace a kultivace COCs

Oocyty byly získávány z vaječníků dosud necyklujících poražených prasniček. Kumulo-oocytární komplexy (COCs) byly izolovány aspirací z folikulů o průměru 2 až 5 mm pomocí injekční stříkačky a jehly 20G. Pro *in vitro* zrání byly použity jen COCs s neporušenou ooplazmou a kompaktní vrstvou kumulárních buněk. Pro objasnění úlohy kumulu v *in vitro* zrání oocytů byly použity denudované oocyty (DOs) zbavené kumulárních buněk pomocí tenkostěnné kapiláry.

Oocyty byly kultivovány ve 4-jamkových Petriho miskách (Nunc, Denmark) v objemu 1 ml modifikovaného kultivačního média M199 (Sigma-Aldrich, USA), obsahující hydrogenuhličitan sodný (32,5 mM), laktát vápenatý (2,75 mM), gentamicin (0,025 mg/ml), HEPES (6,3 mM), gonadotropní hormony eCG a hCG v poměru 13,5 I.U. : 6,6 I.U./ml (P.G.600; Intervet, Holland) a 5 % (v/v) fetálního bovinního séra (GibcoBRL; Life Technologies, Deutsdsland), v podmínkách řízené atmosféry 5 % CO_2 ve směsi se vzduchem při 39 °C po dobu 12 – 48 hod.

Pro potřeby prodloužené kultivace oocytů byly využity *in vitro* dozrálé oocyty za popsaných podmínek. Další kultivace probíhala v modifikovaném M199 médiu s 5 % fetálního bovinního séra bez gonadotropinů, v podmínkách řízené atmosféry po dobu 24, 48 a 72 hod.

4.2. Hodnocení meiotického zrání oocytů

Oocyty byly po kultivaci zbaveny kumulárních buněk pomocí tenkostěnné kapiláry a montovány na podložní sklo, následně fixovány v octové kyselině a ethanolu (1:3, v/v) min. 48 hod. Pomocí mikroskopu s fázovým kontrastem byla hodnocena stádia meiotického zrání oocytů obarvených 1,0 % orceinem. Bylo rozlišováno pět stádií jaderného zrání oocytů (podle Motlík *et* Fulka, 1976): GV – zárodečný váček; LD – pozdní diakineze; MI – metafáze 1. meiotického dělení; AITI – přechod z anafáze do telofáze 1. meiotického dělení; MII – metafáze 2. meiotického dělení.

4.3. Stanovení aktivity kináz v oocytech

Kinázová aktivita byla měřena podle Kubelky *et al*. (2000). Z kultivovaných oocytů byly připraveny buněčné extrakty pomocí 5µl extrakčního pufru. V extraktech oocytů byla měřena aktivita po fosforylaci specifického substrátu MPF - Histonu H1 (H1), a MAPK - myelinového proteinu (MBP). K fosforylaci substrátů bylo v reakční směsi obsahující

buněčný extrakt, H1 a MBP použito radioaktivně značeného izotopu 500 μCi/ml [γ-^{32}P]ATP (GE Healthcare Life Sciences, UK).

Histon H1 a MBP byly následně separovány pomocí SDS-polyakryamidové elektroforézy na 10 % separačním gelu. Gel byl obarven modří Comassie Blue a vysušen. Pomocí scaneru byla odečtena intenzita signálu fosforylovaných H1 a MBP. Hodnoty aktivity MPF a MAPK v oocytech byly vyjádřeny pomocí denzitometrie fosforylovaných substrátů H1 a MBP a vztaženy relativně k MI-oocytům kontrolní skupiny po 24 hod. kultivace, kde je aktivita obou faktorů nejvyšší (Kubelka *et al*., 2000).

4.4. Imunolokalizace proteinů v oocytech

Oocyty byly po kultivaci zbaveny *zony pellucidy* pomocí 0,1% proteázy ze *Streptomyces griseus*. Oocyty byly fixovány v 2,5% (w/v) paraformaldehydu po dobu 60 min. Poté byly oocyty permeabilizovány v 0,5% Triton X-100 a inkubovány s primární protilátkou specifickou proti lokalizovanému proteinu, v ředění 1:100, při 4°C přes noc. Následně byla použita sekundární protilátka IgG konjugovaná s fluorescein isothiokyanátem (FITC), v ředění 1:100, při laboratorní teplotě 60 min. Chromatin byl obarven pomocí Hoechst 33258. Oocyty byly následně použity pro přípravu preparátů umístěním do montovacího média Vectashield. Oocyty byly nasnímány pomocí skenovacího konfokálního mikroskopu (Leica, Germany). Získané obrázky byly podrobeny analýze obrazu a měření signálu intenzity v programu NIS Elements 3.00 (Laboratory Imaging, Czech Republic). Intenzita signálu byla vyjádřená relativně, vždy k dozrálým MII-oocytům kontrolní skupiny.

4.5. Oocytektomie

Pro hodnocení vlivu oocytu na kumulární expanzi bylo využito postupu oocytektomie. COCs byly oocytektomovány v kultivačním médiu pod minerálním olejem za použití stereomikroskopu osazeného mechanickým mikromanipulátorem (Narishige, Japonsko). COC byl imobilizován fixační kapilárou a oocyt odsát injekční kapilárou za získání oocytektomovaný komplexu (OOX) sestávajícího ze *zony pellucidy* obklopené kumulárními buňkami. OOXs byly kultivovány *in vitro* za stejných podmínek jako COCs.

4.6. Měření kumulární expanze

Jako ukazatel intenzity kumulární expanze bylo využito měření obsahu hyaluronové kyseliny (HA). Po zbavení oocytů expandovaného kumulu po kultivaci COCs byly kumulární buňky expandovaných kumulů vystaveny proteolytickému štěpení za použití proteázy z *Aspergilus*

oryzae 2 hod. a následného přidání proteázy z *Bacillus licheniformis* (Novozymes, Denmark) a další kultivace 3 hod., při 45 °C. Po uvolnění HA do roztoku proteáz následovala centrifugace (5 min. při 10 000 rpm) a odpipetování supernatantu. Koncentrace HA v supernatantu byla měřena spektrofotometricky pomocí QnE Hyaluronic Acid ELISA Assay (Biotech, USA) podle přiloženého protokolu, při 450 nm. Koncentrace HA byla odečítána pomocí kvadratické křivky a hodnoty koncentrace HA byly vyjádřeny relativně a vztaženy k COCs kontrolní skupiny po 48 hod.

4.7. Statistická analýza

Každý experiment byl opakován min. 3x. Výsledky experimentů byly podrobeny statistické analýze v programu SAS 9.1 parametrickým testem ANOVA (t-test). P hodnota menší než 0,05 je považována za statisticky významnou.

5. Výsledky a Diskuze

5.1. Vliv donoru H_2S na meiotické zrání prasečích oocytů

Cílem experimentu bylo zhodnotit vliv Na_2S, exogenního donoru H_2S, na meiotické zrání oocytů v podmínkách *in vitro*. Prasečí oocyty byly kultivovány s donorem H_2S v koncentraci 35 – 300 μM po dobu 20 nebo 30 hod., kdy je možné zachytit všechna stádia meiotického zrání a přesněji hodnotit vliv Na_2S na meiotické zrání. V následujícím experimentu bylo hodnoceno meiotické zrání oocytů zrajících v kultivačním médiu s přídavkem donoru H_2S: 300 μM Na_2S, v časové řadě 12 – 48 hod. po 2 hod.

Experimenty bylo zjištěno, že Na_2S statisticky významně urychluje rozpad zárodečného váčku, znovu zahájení meiotického dělení a přechod do 2. meiotického dělení v závislosti na dávce. Po 20 hod. *in vitro* kultivace dochází k poklesu počtu oocytů setrvávajících ve stádiu GV (31,7 ± 2,9 % u kontrolní skupiny vs. 25,0 ± 4,3 – 20,0 ± 4,3 % pro 70 – 300 μM Na_2S). Současně dochází k nárůstu počtu oocytů, které dosáhly MI (43,3 ± 1,4 % vs. 55,0 ± 2,5 – 72,5 ± 2,5 %). Po 30 hod. meiotického zrání se 70 – 300 μM Na_2S dochází k rychlejšímu dosažení stádií 2. meiotické dělení – AITI (54,2 ± 2,9 % vs. 62,5 ± 4,3 – 69,2 ± 3,8 %). Koncentrace 150 a 300 μM Na_2S navodily po 30 hod. *in vitro* zrání dosažení stádia MII u 11,7 – 17,5 % oocytů.

Po kultivaci prasečích kumulo-oocytárních komplexů (COCs) s Na_2S 12 – 48 hod. bylo pozorováno urychlení meiotického zrání oocytů, kdy docházelo statisticky významně k časnějšímu poklesu počtu oocytů ve stádiu GV ve 14 – 22 hod. *in vitro* zrání. Současně oocyty dosahovaly signifikantně rychleji pokročilejších stádií meiotického zrání, jako je LD a MI. Oocyty kultivované s Na_2S navíc vykazovaly rychlejší přechod do 2. meiotického dělení a dosahovaly tak stádií AITI a MII signifikantně rychleji. Výsledky jsou součástí publikace Nevoral *et al*. (2014), viz Příloha 1.

Donor exogenního H_2S urychluje rozpad zárodečného váčku a znovu zahájení meiotického zrání prasečích oocytů mezi 14 a 22 hod. *in vitro* kultivace. Donor urychluje rovněž dozrání oocytů, dosažení stádia MII a vydělení 1. pólového tělíska o 4 hod. dříve v porovnání s kontrolní skupinou. Zvolené koncentrace donoru H_2S byly použity s ohledem na předchozí práce (Wang, 2002; Shibuya *et al*., 2009) a lze tak předpokládat, že účinek exogenního H_2S na zrající oocyty je fyziologický a spočívá v urychlení aktivace klíčových faktorů meiotického zrání – M-fázi/zrání podporujícího faktoru (MPF) a mitogeny aktivované protein kinázy (MAPK).

5.2. Vliv donoru H_2S na kinázovou aktivitu ve zrajících prasečích oocytech

V následujícím experimentu byl hodnocen vliv 300 μM Na_2S na cytoplazmatické zrání prasečích oocytů vyjádřené aktivitou kináz MPF a MAPK. Aktivita kináz byla měřena v časové řadě po 2 a 6 hod. a vztažena vždy k oocytům kontrolní skupiny po 24 hod. *in vitro* zrání, kdy je předpokládána nejvyšší aktivita obou faktorů (Kubelka *et al*., 2000).

V obou dílčích experimentech byl pozorován nárůst aktivity MPF a MAPK mezi 12 a 24 hod. *in vitro* kultivace. Již po 20 hod. *in vitro* zrání oocytů Na_2S statisticky významně urychlil nástup aktivity MAPK o 19,9 %. Po 22 hod. *in vitro* zrání Na_2S zvýšil aktivitu MPF o 59,9 %, rozdíl je statisticky významný. Viz Příloha 1.

V souladu s předchozími pracemi (Kubelka *et al*., 2000) výsledky experimentů prokázaly, že změny v dynamice meiotického zrání jsou determinovány zvýšenou aktivitou MPF a MAPK v cytoplazmě. Možným vysvětlením pro mechanizmus účinku H_2S je proces sulfhydratace, post-translační modifikace proteinů na reziduech sirných aminokyselin prostřednictvím molekuly H_2S (Mustafa *et al*., 2009a). V somatických buňkách bylo popsáno zapojení H_2S prostřednictvím sulfhydratace do signální kaskády Mos/MEK/MAPK, kdy je sulfhydratován faktor MEK (Zhao *et al*., 2014). Protože se MAPK podílí na další aktivaci MPF následované GVBD (Ohashi *et al*., 2003), lze předpokládat, že sulfhydratace MEK je odpovědná za akceleraci MAPK po 20 hod. *in vitro* zrání oocytů s donorem H_2S a později nepřímo za akceleraci aktivity MPF po 22 hod. *in vitro* kultivace.

Mimoto je pravděpodobné, že se proces sulfhydratace uplatňuje také v regulaci dalších signálních kaskád. Mezi tyto kaskády patří adenylát-cykláza/cAMP/PKA, kdy je donor exogenního H_2S schopen vyvolat nárůst koncentrace buněčného posla cAMP (Njie-Mbye *et al*., 2012). Dalším potenciálním cílem gasotransmiteru H_2S je signální dráha PI3K/Akt (Huang *et al*., 2010). Možnými sulfhydratovanými proteiny jsou také iontové kanály (Tang *et al*., 2010, 2013), jejichž úloha byla popsána v souvislosti se zvýšením koncentrace cAMP v cytoplazmě prostřednictvím donoru H_2S (Njie-Mbye *et al*., 2012). Signální dráhy a pohyb iontů v oocytu se uplatňují v regulaci meiotického zrání (Hu *et al*., 2008; Kalous *et al*., 2009; Du *et al*., 2010) a lze tak předpokládat efekt H_2S v meiotickém zrání prostřednictvím jejich ovlivnění.

5.3. Úloha kumulárních buněk v meiotickém zrání urychleném donorem H_2S

Pro objasnění úlohy kumulárních buněk v meiotickém zrání oocytů urychleném přídavkem 300 μM Na_2S byly kultivovány denudované oocyty (DOs) zbavené kumulárních buněk 20 a 30 hod. po kultivaci v podmínkách *in vitro*.

V souladu s dřívějšími publikacemi (Tanghe *et al*., 2002; Li *et al*., 2008) bylo zjištěno, že zbavení oocytů kumulárních buněk urychluje nástup GVBD a další průběh meiotického zrání. Ve skupině DOs kultivovaných s přídavkem Na_2S došlo k dalšímu urychlení meiotického zrání, kdy u 35,0 % oocytů již po 20 hod. *in vitro* zrání došlo k zahájení 2. meiotického dělení a dosažení stádia AITI. Po 30 hod. kultivace bylo pozorováno další signifikantní urychlení meiotického zrání DOs ošetřených Na_2S, kdy stádia AITI dosáhlo 39,2 % a MII 30,0 % oocytů, viz Příloha 1.

Výsledky demonstrují, že přítomnost kumulárních buněk potlačuje urychlující efekt Na_2S na meiotické zrání oocytů. Lze předpokládat, že exogenní H_2S působí na meiotické zrání oocytů přímo, bez zapojení buněčné signalizace kumulárních buněk. Pradĕpodobně je H_2S schopen potlačit již dříve popsaný inhibující efekt přítomnosti kumulárních buněk na zrání oocytů (Tanghe *et al*., 2002).

5.4. Vliv donoru H_2S na kumulární expanzi prasečích COCs

Cílem experimentu bylo hodnocení kumulární expanze podle obsahu hyaluronové kyseliny (HA) v prasečích COCs kultivovaných 48 hod. s různou koncentrací Na_2S a v COCs zrajících 12 – 48 hod. s 300 μM Na_2S. Obsah HA byl vyjádřen jako HA zadržená v COCs a celková HA zahrnující HA zadrženou v COCs a HA uvolněnou do kultivačního média. Obsah HA byl vyjádřen relativně vždy k celkové produkci HA v COCs kontrolní skupiny dozrálých po 48 hod., kdy je obsah HA nejvyšší.

Bylo zjištěno, že celková produkce HA je donorem H_2S potlačena statisticky významně o 21,9 - 31,3 % po 48 hod. *in vitro* kultivace. Zadržená HA v COCs není ovlivněna přídavkem donoru H_2S v jakékoliv koncentraci. V časové řadě po 12 hod. dochází k statisticky významnému potlačení celkové produkce HA po 36 hod. (62,4 ± 0,4 % vs. 48,9 ± 4,2 %) a 48 hod. (100,0 ± 0,0 % vs. 70,9 ± 12,0 %) *in vitro* kultivace, Viz Příloha 1.

Vysvětlením pro inhibiční efekt exogenního H_2S může být zapojení tohoto gasotransmiteru do signálních drah PI3K/Akt a cAMP/PKA (Huang *et al*., 2010; Njie-Mbye *et al*., 2012), které se podílí na regulaci kumulární expanze (Downs *et* Hunzicker-Dunn, 1995; Hoshino *et al*., 2004). Potlačená kumulární expanze donorem H_2S nezpůsobila neúspěch meiotického zrání, narozdíl od dříve publikovaných výsledků, kde byla demonstrována pozitivní korelace mezi intenzitou kumulární expanze a úspěšností meiotického zrání (Qian *et al*., 2003). Důvodem snížení intenzity kumulární expanze v druhé polovině meiotického zrání v *in vitro* podmínkách může být snížená potřeba pokračující kumulární expanze u rychleji zrajících oocytů.

5.5. Úloha oocytu v kumulární expanzi potlačené donorem H_2S

Pro hodnocení významu přítomnosti oocytu v kumulární expanzi potlačené donorem H_2S bylo využito metody oocytektomie. Experimenty prokázaly, že odstranění oocytu z komplexu způsobuje potlačení kumulární expanze oocytektomovaných komplexů (OOXs) vyjádřené celkovou produkcí HA. Již dříve byl popsán inhibující efekt odstranění oocytu na kumulární expanzi (Nakayama *et al*., 1996; Kimura *et al*., 2002). Obsah HA zadržené v komplexech však není oocytektomií ovlivněn.

Zatímco oocytektomie neovlivňuje obsah zadržené HA v komplexech kultivovaných v čistém kultivačním médiu, statisticky významně je potlačena produkce HA zadržené v OOXs kultivovaných v přítomnosti 300 μM Na_2S (42,6 ± 7,1 % u COCs kultivovaných s Na_2S vs. 22,4 ± 6,0 % u OOXs s Na_2S). Současně celková produkce HA a obsah HA zadržené v OOXs nejsou ovlivněny přítomností Na_2S, viz Příloha 1. Oocyt tak zjevně sehrává úlohu při regulaci kumulární expanze v přítomnosti donoru exogenního H_2S. Za potenciální cíl H_2S může být považován zejména diferenciační růstový faktor 9 (GDF9), který se významně uplatňuje ve stimulaci kumulární expanze oocytem prostřednictvím regulace aktivity HA syntázy 2 v kumulárních buňkách (Dragovic *et al*., 2005; Procházka *et al*., 2012). Tento předpoklad je v souladu se zjištěním, že exprese růstových faktorů z rodiny TGFβ je ovlivněna donorem H_2S v somatických buňkách (Mard *et al*., 2012).

5.6. Vliv donoru H_2S na časný embryonální vývoj prasete

Cílem bylo hodnocení vývojové kompetence oocytů dozrálých v *in vitro* podmínkách s přídavkem donoru H_2S: 300 μM Na_2S. Dozrálé oocyty ošetřené Na_2S byly aktivovány po 44 hod. a oocyty kontrolní skupiny po 46 hod., tedy když 100 % kultivovaných oocytů dozrálo do stádia MII. Jako hodnotící kritérium bylo použito míry aktivace a časného embryonálního vývoje po partenogenetické aktivaci 25μM Ca^{2+}-ionoforem.

Bylo zjištěno, že donor H_2S statisticky významně zvyšuje schopnost aktivace oocytů (91,7 ± 3,2 % u oocytů dozrálých s Na_2S vs. 75,8 ± 3,2 % u oocytů kontrolní skupiny). Počet dvoubuněčných embryí po 48 hod. kultivace zygot, počet morul a blastocyst po 7 dnech kultivace zygot nebyl ovlivněn ošetřením donoru H_2S během *in vitro* zrání, viz Příloha 1.

Výsledky partenogenetického vývoje oocytů dozrálých s Na_2S podporují domněnku, že popsané účinky exogenního donoru H_2S – urychlené meiotické zrání a potlačená kumulární expanze, jsou fyziologické. Mimo dosud sledovaných popsaných akcelerujících účinků donoru H_2S na meiotické zrání oocytů lze očekávat protektivní efekt tohoto gasotransmiteru

ve zrajících oocytech prasete. Vysvětlením může být sulfhydratace zmíněné kinázy MEK1, která je prostřednictvím MAPK odpovědná za aktivaci poly(ADP-ribóza) polymerázy 1 (PARP1) (Kauppinen *et al*., 2006; Zhao *et al*., 2014), jejíž úloha spočívá v reparaci zlomů DNA (Beck *et al*., 2014). Rovněž byl popsán protektivní účinek H_2S, který spočívá v potlačení oxidačního stresu za zvýšené exprese markerů buněčného zdraví (Perrino *et al*., 2009; Osborne *et al*., 2010) a aktivace $NADP^+$-dependentních histon deacetyláz – sirtuinů (Shang *et al*., 2012; Suo *et al*., 2013). Výsledkem možného ovlivnění těchto signálních drah je v experimentech popsaná zvýšená schopnost aktivace oocytů dozrálých v přítomnosti donoru H_2S.

5.7. Vliv inhibice c-Jun N-terminální kinázy na průběh stárnutí prasečích oocytů

Cílem experimentů popisujících úlohu c-Jun N-terminální kinázy (JNK) ve stárnoucích oocytech prasete bylo zhodnotit účinek 1,9-parazoloanthronu, specifického inhibitoru JNK. Efekt inhibitoru byl vyjádřen schopností aktivace, časného embryonálního vývoje, fragmentace DNA a expresí pro-apoptotického faktoru Bax po 24 hod. prodloužené kultivace s inhibitorem JNK.

Experimenty prokázaly, že použití aktivního inhibitoru JNK má za následek statisticky významné potlačení programované buněčné smrti a lýzy oocytů stárnoucích 48, 72 a 96 hod. Rovněž byl prokázán protektivní účinek inhibitoru JNK, který potlačuje fragmentaci DNA o 51,4 %, rozdíl je statisticky signifikantní. Současně dochází k významnému zvýšení počtu aktivovaných oocytů během stárnutí po inhibici JNK. Současně však bylo zjištěno, že inhibice JNK statisticky významně snižuje podíl Ca^{2+}-ionoforem aktivovaných oocytů schopných dosáhnout dalších stupňů embryonálního vývoje, jako je 2-buněčné embryo a blastocysta. Množství pro-apoptotického faktoru Bax ve stárnoucích oocytech nebylo ošetřením inhibitoru JNK ovlivněno. Výsledky jsou publikovány v práci Sedmíková *et al*. (2013), viz Příloha 3.

Provedenými experimenty bylo zjištěno, že již po 24 hod. prodloužené kultivace po inhibici JNK došlo k potlačení fragmentace DNA následované morfologickými projevy stárnoucích oocytů, které vykazovaly nižší míru buněčné smrti. Zjištění je v souladu s předchozími publikacemi popisující JNK jako klíčový regulační faktor programované buněčné smrti v somatických buňkách (Dhanasekaran *et* Reddy, 2008; Plotnikov *et al*., 2001), který je odpovědný za fragmentaci DNA (Chen *et al*., 2003a). Absence účinku inhibitoru JNK po 48 – 96 hod. prodloužené kultivace je zřejmě způsobená dalšími faktory nezávislými na JNK a mitochondriální dráze indukující programovanou buněčnou smrt. Stimulem pro aktivaci pro-apoptotických mechanizmů jsou reaktivní formy kyslíku (ROS), jejichž koncentrace se s

prodlouženou kultivací ve stárnoucích oocytech zvyšuje (Miao *et al*., 2009). Zvýšená koncentrace ROS je schopná vyvolat programovanou buněčnou smrt, mimo aktivace JNK, také prostřednictvím endoplazmatického retikula, ovlivňujícího balanci iontů způsobenou vyplavením Ca^{2+} (Takahashi *et al*., 2003; He *et al*., 2008). Lze předpokládat, že inhibice JNK způsobuje zvýšení koncentrace Ca^{2+} iontů v cytoplazmě, což vede k vyššímu počtu aktivovaných oocytů (Wassarman, 1988; Yanagimachi, 1988).

Je zřejmé, že ve stárnoucích prasečích oocytech nedochází k zapojení Bax do signální dráhy JNK. Toto zjištění neodpovídá mechanizmům popsaným v somatických buňkách, kde JNK způsobuje fragmentaci DNA prostřednictvím pro-apoptotických faktorů Bax a Bad (Antignani *et* Youle, 2006; Chu *et al*., 2009), ani zvýšené expresi Bax po indukci apoptózy oocytů *Xenopus laevis* pomocí JNK (Du Pasquier *et al*., 2011). Vysvětlením může být zapojení dalších pro-apoptotických faktorů v indukci buněčné smrti stárnoucích prasečích oocytů, kde kandidátním faktorem může být Bad (Bhakar *et al*., 2003). Zapojení alternativních signálních drah nezávislých na JNK je patrně příčinou snížené vývojové schopnosti navzdory inhibici JNK. Mimoto, indukce buněčného stresu prostřednictvím zapojení endoplazmatického retikula, v kterém zřejmě inhibice JNK sehrává roli, může vést k dalšímu zhoršení časného embryonálního vývoje (Zhang *et al*., 2012).

5.8. Přítomnost NO syntáz a jejich inhibice ve stárnoucích prasečích oocytech

Cílem experimentu bylo prokázat přítomnost jednotlivých izoforem NO syntáz (NOS) ve stárnoucích prasečích oocytech. Následně byla hodnocena úloha NOS za použití inhibitorů L-NAME nebo aminoguanidinu.

V dozrálých MII-oocytech a v oocytech stárnoucích 24 – 72 hod. byla pomocí imunolokalizace prokázána přítomnost všech tří izoforem NOS: eNOS, iNOS, nNOS. Sledované izoformy vykazovaly různou intracelulární distribuci: izoforma eNOS byla distribuována rovnoměrně v cytoplazmě, iNOS bezprostředně pod cytoplazmatickou membránou a nNOS zejména v prvojádrech spontánně aktivovaných oocytů. Současně byla provedena kvantifikace signálu, která prokázala, že intenzita signálu a předpokládané množství iNOS a nNOS během stárnutí klesá o 88 %, resp. 63 %, již po 24 hod. prodloužené kultivace. Intenzita signálu eNOS zůstává po 48 hod. prodloužené kultivace bez změny a klesá statisticky významně o 50 % po 72 hod. Inhibitory L-NAME a aminoguanidin potlačují programovanou buněčnou smrt a lýzu po 72 hod. prodloužené kultivace v závislosti na dávce inhibitoru. Stárnoucí oocyty tak vykazovaly vyšší výskyt intaktních oocytů v MII

stádiu a spontánně partenogeneticky aktivovaných oocytů. Výsledky jsou součástí publikace Nevoral *et al*. (2013), viz Příloha 4.

Prokázaná přítomnost izoforem NOS v oocytech prasete je v souladu s dříve publikovanými pracemi (Tao *et al*., 2005a; Chmelíková *et al*., 2010; Tichovská *et al*., 2011). Lokalizace eNOS a iNOS v cytoplazmě a v blízkosti cytoplazmatické membrány oocytu patrně souvisí s vazbou těchto izoforem na komponenty cytoskeletu (Brophy *et al*., 2000; Zeng *et* Morrison, 2001). Přítomnost izoformy nNOS v prvojádrech aktivovaných oocytů je ve shodě s již popsanou lokalizací v jádrech somatických buněk (Yuan *et al*., 2004) a je pravděpodobné, že přítomnost v prvojádrech zygot souvisí s transkripční aktivitou po reaktivaci embryonálního genomu (Latham *et* Schultz, 2001). Pomalejší pokles intenzity signálu eNOS je vysvětlován vyšším množstvím této izoformy v dozrálém oocytu v porovnání s izoformami iNOS a nNOS a různým poločasem rozpadu jednotlivých izofrem (Rämet *et al*., 2003; Kim *et al*., 2005; Hattori *et* Tabata, 2006). Inhibovaná produkce NO ve stárnoucích oocytech vedla k potlačení buněčné smrti, podobně jako v případě Langerhansových ostrůvků a β-buněk slinivky (Corbett *et* McDaniel, 1996). Lze předpokládat, že vyšší míra spontánně aktivovaných oocytů je výsledkem spíše potlačení buněčné smrti než přímého efektu absence NO v oocytech.

6. Závěr

Meiotické zrání oocytů je klíčovým procesem pro reprodukční biotechnologie, jako je *in vitro* oplození (IVF) nebo přenos jader somatických buněk (SCNT). Detailní studium meiotického zrání oocytů, stárnutí oocytů a testování potenciálních látek s protektivním účinkem je nezbytné pro vyšší efektivitu získávání *in vitro* dozrálých oocytů a tedy další pokrok reprodukčních biotechnologií. Cílem práce bylo ověřit hypotézu, že gasotransmitery sehrávají v prasečích oocytech fyziologickou úlohu v regulaci meiotického zrání, kumulární expanze a stárnutí během prodloužené kultivace.

Experimenty bylo zjištěno, že donor gasotransmiteru H_2S signifikantně urychluje meiotické zrání prasečích oocytů, kdy oocyty rychleji podstupují rozpad zárodečného váčku (GVBD) a dosažení metafáze II (MII). Bylo ověřeno, že urychlení meiotického zrání je výsledkem akcelerované aktivace klíčových faktorů meiotického zrání – M-fázi/zrání podporujícího faktoru (MPF) a mitogeny aktivované protein kinázy (MAPK). Současně bylo zjištěno, že se přítomnost kumulárních buněk se na akceleraci zrání oocytů nepodílí a oocyty zbavené kumulárních buněk (DOs) a současně ošetřené donorem exogenního H_2S zrají rychleji v porovnání s kumulo-oocytárními komplexy (COCs).

Produkce hyaluronové kysleiny (HA) a intenzita kumulární expanze byly donorem H_2S během kultivace *in vitro* potlačeny v druhé polovině meiotického zrání oocytů. Výsledky podporují domněnku, že v potlačení kumulární expanze donorem H_2S sehrává úlohu oocyt a exogenní H_2S působí prostřednictvím inhibice oocytárních růstových faktorů fyziologicky stimulující kumulární expanzi.

Současně bylo zjištěno, že dozrálé oocyty s donorem H_2S vykazovaly vyšší míru partenogenetické aktivace, přičemž použitý donor H_2S neovlivnil dosažení dalších vývojových stádií časného embryonálního vývoje.

Další experimenty byly zaměřené na regulační faktory stárnutí oocytů. Mezi nimi byla studována úloha c-Jun N-terminální kinázy (JNK) během prodloužené kultivace. Výsledky dokazují zapojení této kinázy do procesů stárnutí a příznivé účinky její inhibice na fragmentaci DNA a časný embryonální vývoj. Je zřejmé, že další faktory nezávislé na signalizaci JNK jsou zapojeny do regulace stárnutí a jsou tak alternativními způsoby indukující buněčnou smrt oocytů po prodloužené kultivaci *in vitro*.

Během stárnutí ocoytů byl studován rovněž vliv gasotransmiteru NO, který je v buňkách produkován NO syntázami (NOS). Přítomnost všech tři izoforem NOS byla prokázána v dozrálých a stárnoucích oocytech, včetně jejich specifické intracelulární distribuce.

Po inhibici fyziologické produkce NO bylo sledováno potlačení programované buněčné smrti oocytů a lýzy a současně vyšší podíl oocytů spontánně partenogeneticky aktivovaných.

Výsledky práce poukazují na gasotransmitery H_2S a NO jako na fyziologické regulátory uplatňující se v procesech meiotického zrání a stárnutí během prodloužené kultivace. Dosavadní poznání poukazuje na možnosti dalšího studia zejména v oblasti embryonálního vývoje a molekulárních mechanizmů účinků H_2S. Studium těchto signálních molekul s protektivním účinkem pomůže optimalizaci podmínek *in vitro* pro produkci kvalitních a životaschopných embryí.

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8. Přílohy

8.1. Příloha 1: Nevoral *et al*. (2014): Dual effect of hydrogen sulfide donor on meiosis and cumulus expansion of porcine cumulus-oocyte complexes. PLOS ONE, 9: e99613.

Cílem této práce bylo ověřit hypotézu, že gasotransmiter H_2S je zapojen do regulace meiotického zrání oocytů a kumulární expanze kumulo-oocytárních komplexů (COCs). Pro ověření hypotézy byly prasečí COCs kultivovány s donorem exogenního H_2S: Na_2S. V experimentech byl sledován průběh meiotického zrání oocytů, aktivita klíčových faktorů MPF a MAPK, kumulární expanze a časný embryonální vývoj oocytů zrajících v přítomnosti donoru H_2S. Bylo zjištěno, že donor H_2S urychluje GVBD, další průběh meiotického zrání a dosažení stádia MII, jako výsledek předchozí akcelerace kinánzové aktivity MPF a MAPK. Přítomnost kumulárních buněk akcelerační efekt donoru H_2S potlačuje. Donor H_2S současně potlačuje kumulární expanzi, patrně prostřednictvím potlačení oocytární sekrece růstových faktorů podporující kumulární expanzi. Pozitivní efekt exogenního H_2S byl ověřen po partenogenetické aktivaci hodnocením vývojové kompetence získané během meiotického zrání s donorem H_2S.

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

Jan Nevoral[1☯]*, Jaroslav Petr[2☯], Armance Gelaude[3], Jean-Francois Bodart[3], Veronika Kucerova-Chrpova[1], Marketa Sedmikova[1], Tereza Krejcova[1], Tereza Kolbabova[1¤], Marketa Dvorakova[1], Alena Vyskocilova[1], Ivona Weingartova[1], Lenka Krivohlavkova[1], Tereza Zalmanova[1], Frantisek Jilek[1]

1 Department of Veterinary Sciences, Czech University of Life Sciences in Prague, Prague, Czech Republic, **2** Research Institute of Animal Production, Prague, Czech Republic, **3** Laboratoire de Régulation des Signaux de Division – EA 4479, Université Lille1, Sciences et Technologies, IFR 147, Villeneuved'Ascq Cedex, France

Abstract

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

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* E-mail: nevoral@af.czu.cz

¤ Current address: Department of Game Management and Wildlife Biology, Czech University of Life Sciences in Prague, Prague, Czech Republic

☯ These authors contributed equally to this work.

Introduction

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

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

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

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

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

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

Materials and Methods

In Vitro Oocyte Cultivation with H_2S Donor

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

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

Evaluation of Oocyte Meiotic Maturation

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

Histone H1 and Myelin Basic Protein Double Assay

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

Oocytectomy and OOXs Cultivation

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

Hyaluronic Acid Assay

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

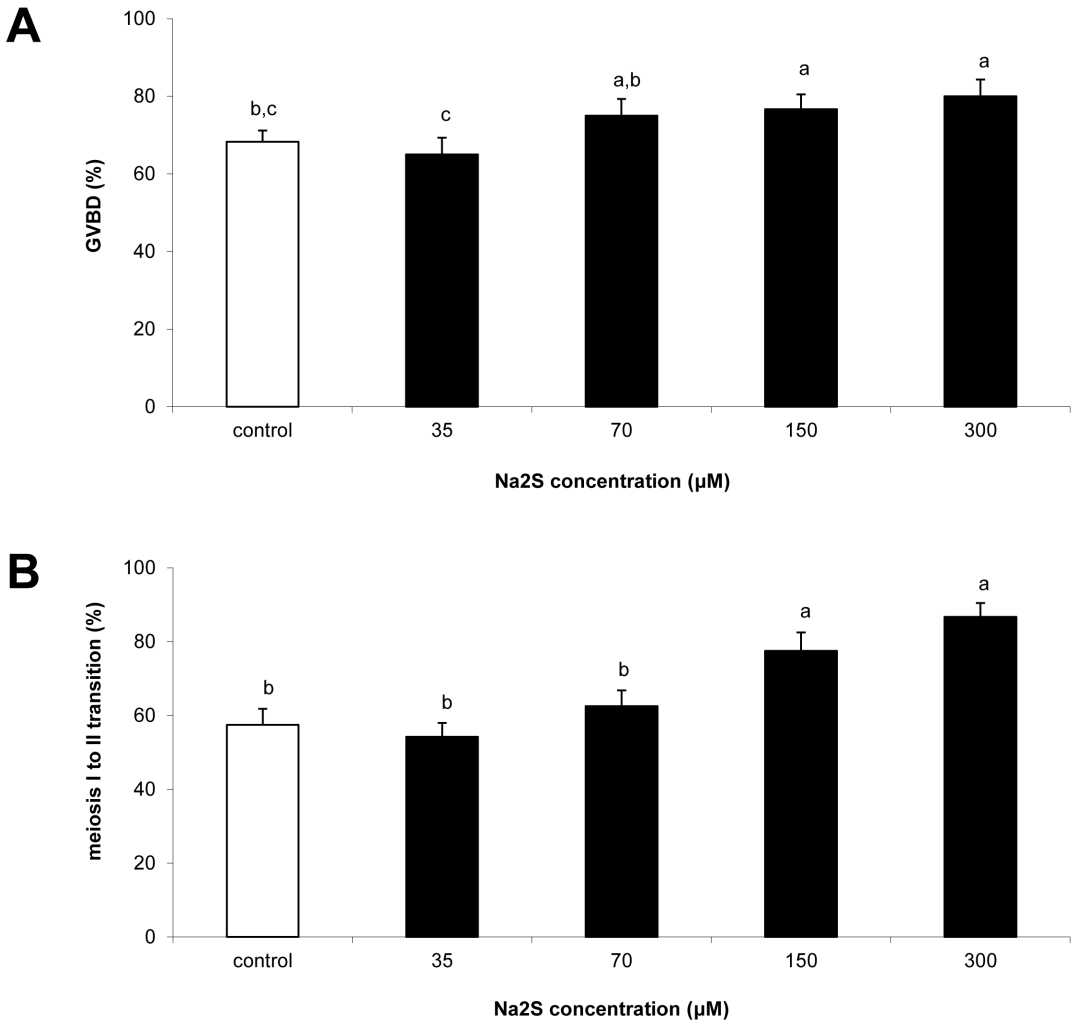

Figure 1. Effect of different Na_2S concentrations on meiosis resumption and transition to meiosis II in oocytes. Proportion of GVBD (A) and meiosis I to II transition (B) during *in vitro* cultivation after 20 and 30 h *in vitro* cultivation, respectively. [a,b,c]Statistically significant differences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

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

Statistical Analysis

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

Results

H_2S Donor Accelerates Oocyte Maturation in a Dose-Dependent Manner

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

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

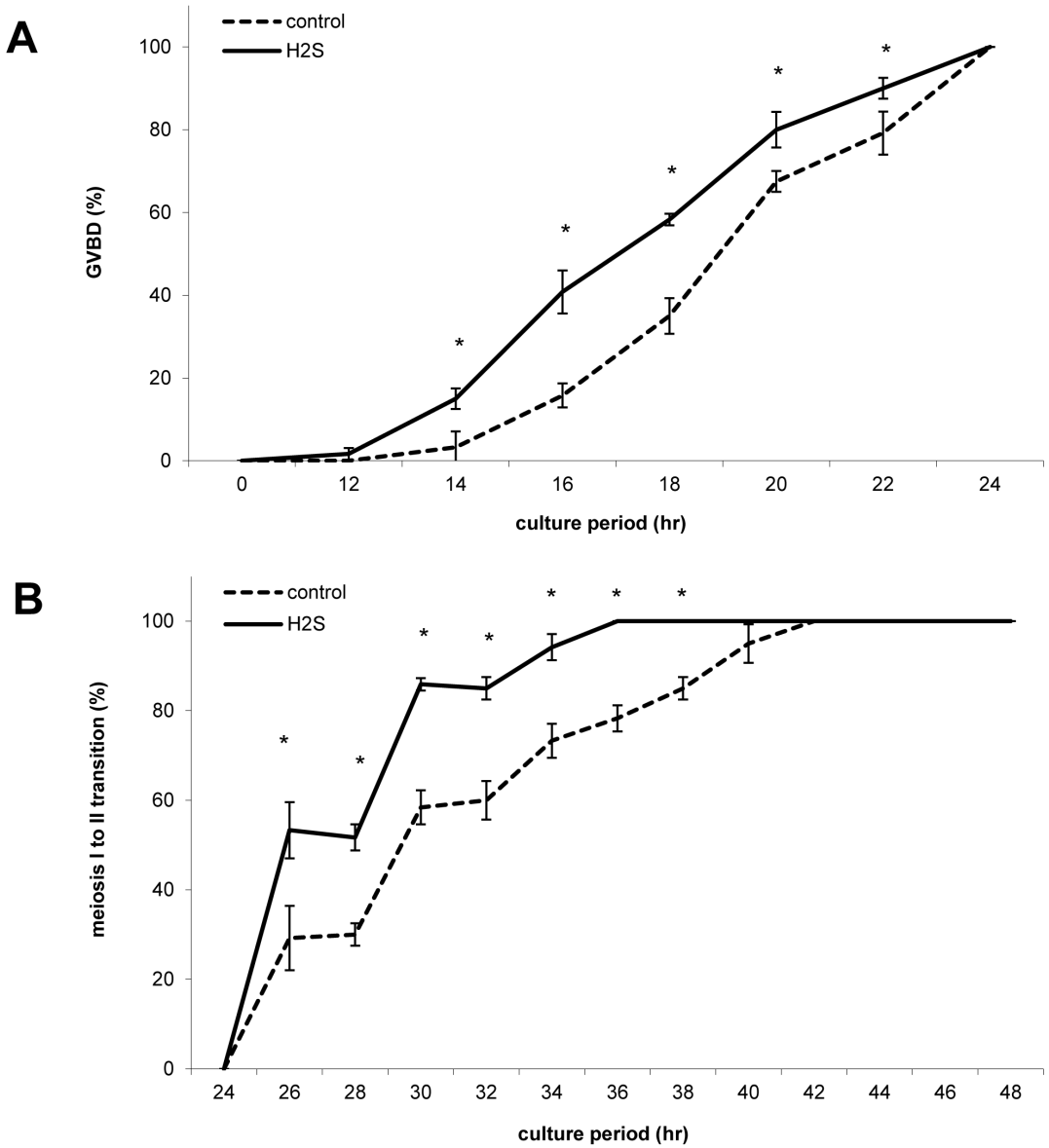

Figure 2. Effect of Na_2S on meiotic resumption and transition to meiosis II during oocyte cultivation. Proportion of GVBD (A) and meiosis I to II transition (B) in oocytes during *in vitro* cultivation over 2 h time scale. H_2S: 300 μM Na_2S. *Statistically significant differences between control and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

H_2S Donor Accelerates Porcine Oocyte Maturation

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

MPF and MAPK Activity Profiles Are Accelerated by H_2S Donor

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

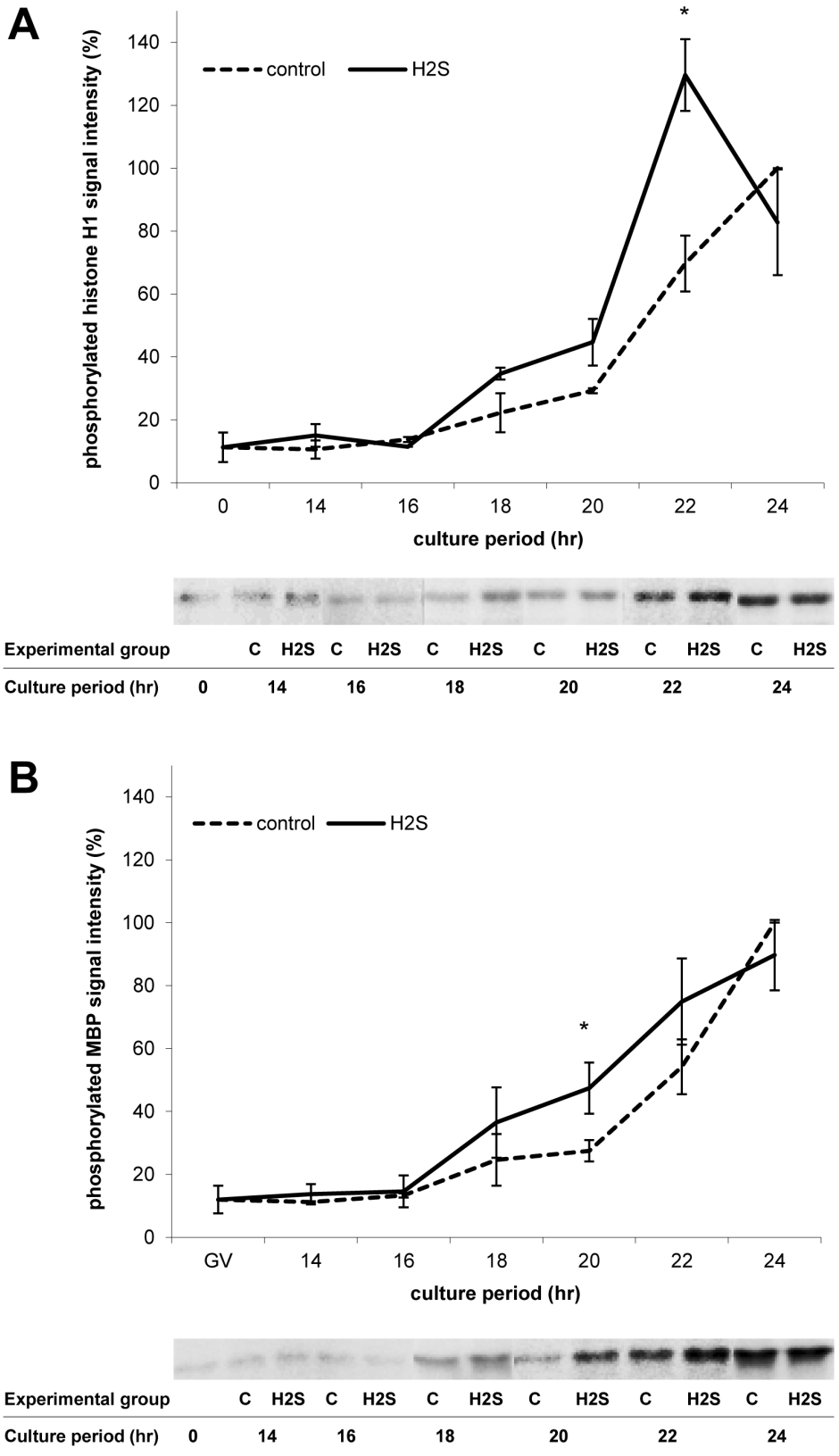

Figure 3. Effect of Na_2S on MPF and MAPK activities during oocyte cultivation. Representative autoradiograms and signal quantifications of phosphorylated histone H1 (A) and MBP (B) reflecting MPF and MAPK activity, respectively. Kinase activity was measured in oocytes cultivated with or without Na_2S over 2 h time scale. The kinase activity was related to oocytes cultivated for 24 hs. C: control; H_2S: 300 μM Na_2S. *Statistically significant differences between control and H_2S groups ($P<0.05$).
doi:10.1371/journal.pone.0099613.g003

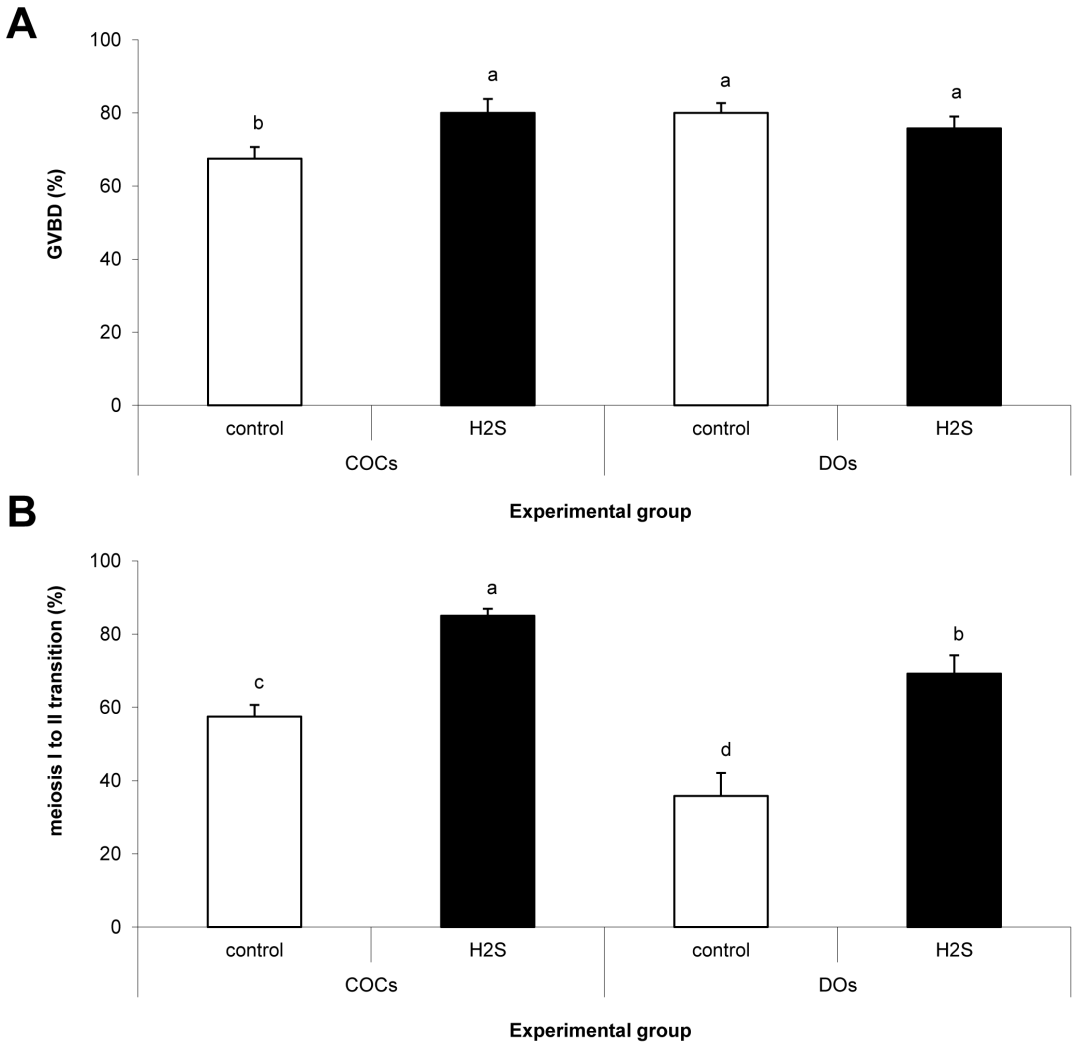

Figure 4. Effect of Na_2S on meiosis resumption and transition to meiosis II in DOs. Proportion of GVBD (A) and meiosis I to II transition (B) during *in vitro* cultivation after 20 and 30 h *in vitro* cultivation, respectively. H_2S: 300 μM Na_2S. [a,b,c]Statistically significant differences among experimental groups ($P<0.05$).
doi:10.1371/journal.pone.0099613.g004

in vitro maturation, significant acceleration of MPF occurred after 22 h cultivation.

H_2S Donor Can Substitute for the Absence of Cumulus Cells

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

H_2S Donor Influences Cumulus Expansion with Presence of Oocytes

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

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

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

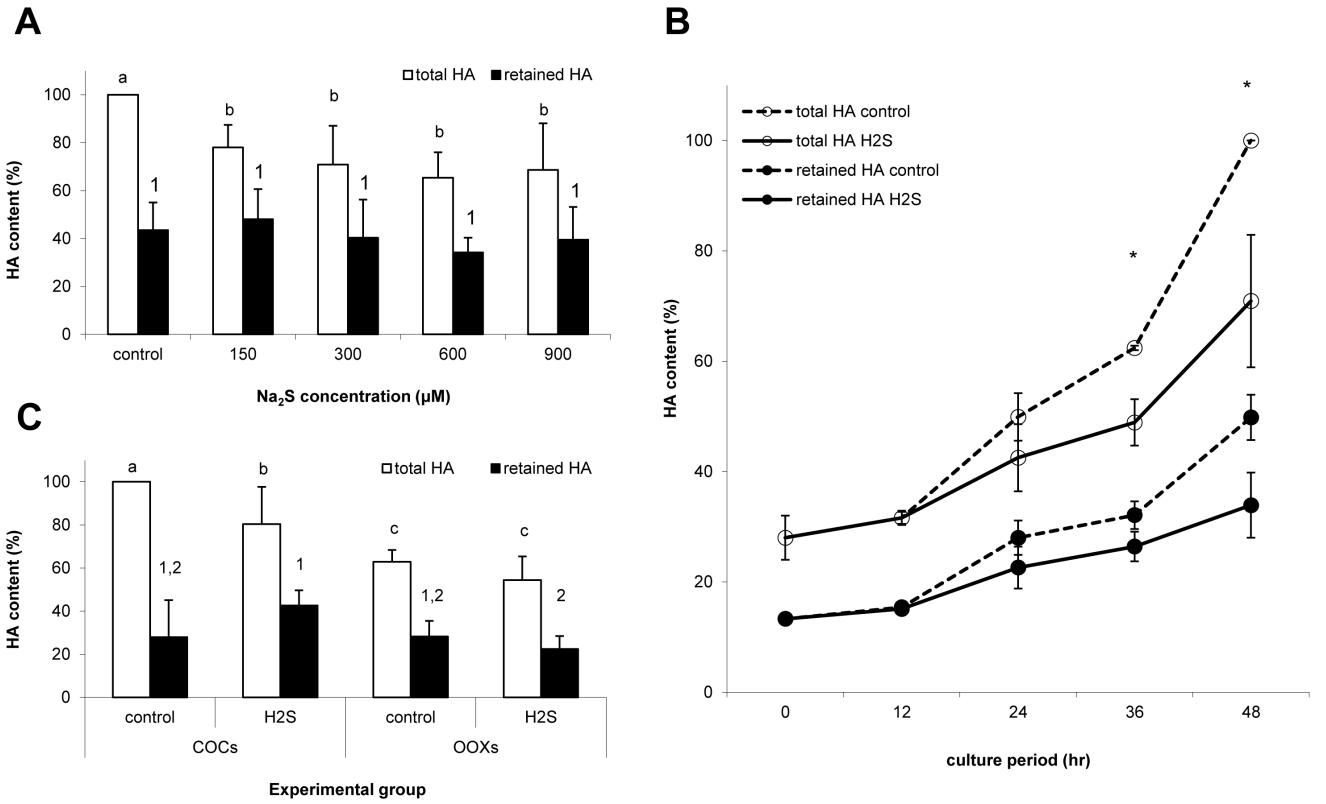

Figure 5. Effect of Na_2S on HA content in expanded cumulus. (A) Total and retained HA content in COCs cultivated with 150–900 μM Na_2S for 48 hs, total HA is related to the control group. (B) Total and retained HA content in COCs during *in vitro* cultivation with 300 μM Na_2S over 12 h time scale, total HA is related to the control group after 48 h cultivation. (C) Total and retained HA content in COCs and OOXs cultivated with or without H_2S donor, total HA is related to the control group of COCs. H_2S: 300 μM Na_2S. [a,b,c]Statistically significant differences among experimental groups in total HA, [1,2]statistically significant differences among experimental groups in retained HA, *statistically significant differences in total HA between control and H_2S groups ($P<0.05$).
doi:10.1371/journal.pone.0099613.g005

cultivated with the H_2S donor for 48 hs. It was found that oocytectomisation reduced total HA in OOXs cultivated in a pure medium by 37.0%. HA production by OOXs cultivated with H_2S donor decreased with no statistical significance in comparison with the above-mentioned OOXs. The data are shown in Figure 5C.

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

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

Discussion

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

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

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

Oocytes were matured with or without Na_2S and partenogenetically activated using calcium ionophore. Pronucleus formation after 24 h zygote culture, cleavage rate after 2 days and blastocyst achievement after 7 days presumptive embryos culture were evaluated (%±SE).
H_2S: 300 μM Na_2S during oocyte maturation.
*Statistically significant differences between control and H_2S group – in column ($P<0.05$).
doi:10.1371/journal.pone.0099613.t001

physiological values in tissues [2,3] and we could assume that the observed effects of H_2S donor exogenously added were not a result of its toxicity but rather relied on the physiological effect of H_2S as a gasotransmitter. To the best of our knowledge, this study is the first one to describe the influence of the H_2S donor on meiotic maturation of oocytes.

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

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

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

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

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

Supporting Information

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

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

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

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

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Author Contributions

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

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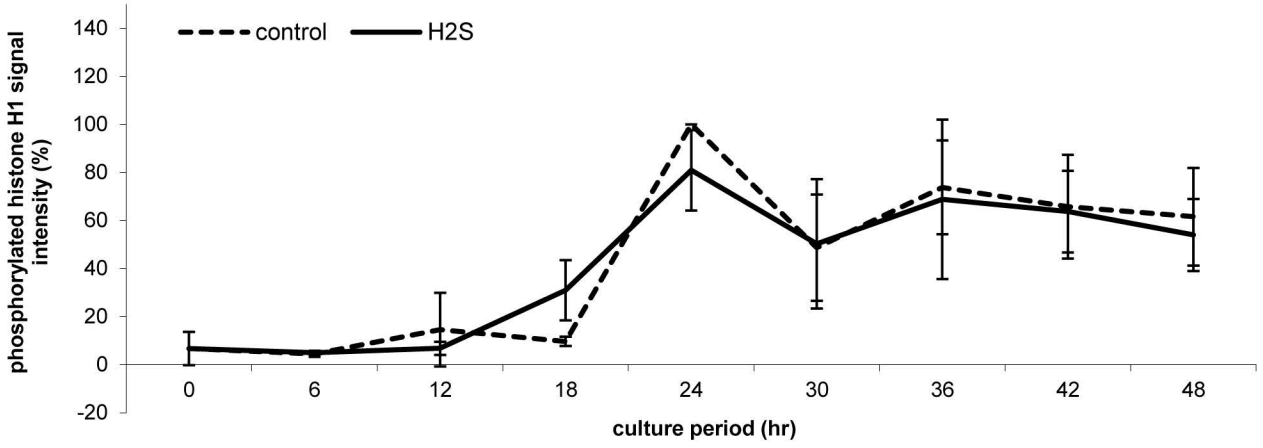
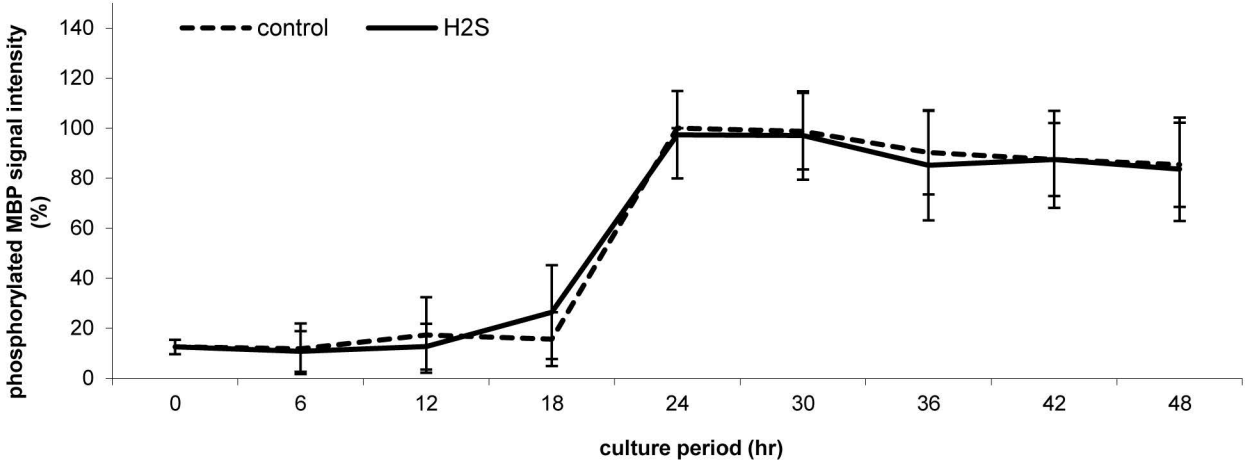
A



Experimental group		C	H2S	C	H2S	C	H2S	C	H2S	C	H2S	C	H2S	C	H2S	C	H2S
Culture period (hr)	0	6		12		18		24		30		36		42		48	

B



Experimental group		C	H2S	C	H2S	C	H2S	C	H2S	C	H2S	C	H2S	C	H2S	C	H2S
Culture period (hr)	0	6		12		18		24		30		36		42		48	

Supporting Information~~s~~ S1

~~Supporting Information~~Table S1a. Effect of different Na_2S concentrations on oocyte maturation after 20 hr cultivation.

	Stage of meiotic maturation (% ± SE)					n
	GV	LD	MI	AI/TI	MII	
control	31.7±2.9^{a,b}	25.0±2.5^{a}	43.3±1.4^{d}	-	-	120
35µl	35.0±4.3^{a}	25.0±4.3^{a}	40.0±0.0^{d}	-	-	120
70µl	25.0±4.3^{b,c}	20.0±5.0^{a,b}	55.0±2.5^{c}	-	-	120
150µl	23.3±3.8^{c}	16.7±1.4^{b}	60.0±4.3^{b}	-	-	120
300µl	20.0±4.3^{c}	7.5±2.5^{c}	72.5±2.5^{a}	-	-	120

GV: germinal vesicle oocytes; LD: late diakinesis ooccytes; MI: metaphase I oocytes; AI/TI: anaphase I to telophase I transition oocytes; MII: metaphase II oocytes. ^{a,b,c,d}Statistically significant differences among experimental groups in the same nuclear stage – in column ($P<0.05$).

~~Supporting Information~~Table S1b. Effect of different Na_2S concentrations on oocyte maturation after 30 hr cultivation.

	Stage of meiotic maturation (% ± SE)					n
	GV	LD	MI	AI/TI	MII	
control	-	-	42.5±4.3^{a,b}	54.2±2.9^{b}	3.3±1.4^{c}	120
35µl	-	-	45.8±3.8^{a}	54.2±3.8^{b}	0.0±0.0^{c}	120
70µl	-	-	37.5±4.3^{b}	62.5±4.3^{a}	0.0±0.0^{c}	120
150µl	-	-	22.5±5.0^{c}	65.8±3.8^{a}	11.7±1.4^{b}	120
300µl	-	-	13.3±3.8^{d}	69.2±3.8^{a}	17.5±6.6^{a}	120

GV: germinal vesicle oocytes; LD: late diakinesis ooccytes; MI: metaphase I oocytes; AI/TI: anaphase I to telophase I transition oocytes; MII: metaphase II oocytes. [a,b,c,d]Statistically significant differences among experimental groups in the same nuclear stage – in column ($P<0.05$).

Supporting Informations S2

~~Supporting Information~~Table S2. Effect of 300μM Na_2S on oocyte maturation.

		Stage of meiotic maturation (% ± SE)					
		GV	LD	MI	AI/TI	MII	n
12hr	control	100±0.0	0.0±0.0	-	-	-	120
	H_2S	98.3±1.4	1.7±1.4	-	-	-	120
14hr	control	96.7±3,8	3.3±3.8	0.0±0.0	-	-	120
	H_2S	85.0±2.5*	0.0±0.0	15.0±2.5*	-	-	120
16hr	control	84.2±2.9	0.0±0.0	15.8±2.9	-	-	120
	H_2S	59.2±5.2*	5.0±2.5*	35.8±5.2*	-	-	120
18hr	control	65.0±4.3	10.8±1.4	24.2±2.9	-	-	120
	H_2S	41.7±1.4*	25.8±3.8*	32.5±5.0	-	-	120
20hr	control	32.5±2.5	25.0±4.3	42.5±2.5	-	-	120
	H_2S	20.0±4.3*	6.7±2.9*	73.3±1.4*	-	-	120
22hr	control	20.8±5.2	20.8±3.8	58.3±2.9	-	-	120
	H_2S	10.0±2.5*	15.0±2.5	75.0±2.5*	-	-	120
24hr	control	-	3.3±3.8	96.7±3.8	-	-	120
	H_2S	-	0.0±0.0	100±0.0	-	-	120
26 h	control	-	-	70.8±7.2	29.2±7.2	-	120
	H_2S	-	-	46.7±6.3*	53.3±6.3	-	120
28hr	control	-	-	70.0±2.5	30.0±2.5	-	120
	H_2S	-	-	48.3±2.9*	51.7±2.9*	-	120
30hr	control	-	-	41.7±3.8	54.2±5.2	4.2±1.4	120
	H_2S	-	-	14.2±1.4*	71.7±3.8*	14.2±2.9*	120
32hr	control	-	-	40.0±4.3	50.0±5.0	10.0±2.5	120

	H_2S	-	-	15.0±2.5*	70.0±4.3*	15.0±2.5	120
34hr	control	-	-	26.7±3.8	42.5±5.0	30.8±3.8	120
	H_2S	-	-	5.8±2.9*	56.7±2.9*	37.5±0.0*	120
36hr	control	-	-	21.7±2.9	40.0±4.3	38.3±3.8	120
	H_2S	-	-	0.0±0.0*	37.5±6.6	62.5±6.6*	120
38hr	control	-	-	15.0±2.5	34.2±3.8	50.8±1.4	120
	H_2S	-	-	0.0±0.0*	23.3±5.2*	76.7±5.2*	120
40hr	control	-	-	5.0±4.3	35.8±1.4	59.2±3.8	120
	H_2S	-	-	0.0±0.0	8.3±1.4*	91.7±1.4*	120
42hr	control	-	-	-	35.8±2.9	64.2±2.9	120
	H_2S	-	-	-	6.7±2.9*	93.3±2.9*	120
44hr	control	-	-	-	23.3±1.4	76.7±1.4	120
	H_2S	-	-	-	0.0±0.0*	100±0.0*	120
46hr	control	-	-	-	-	100±0.0	120
	H_2S	-	-	-	-	100±0.0	120
48hr	control	-	-	-	-	100±0.0	120
	H_2S	-	-	-	-	100±0.0	120

H_2S: 300μM Na_2S. GV: germinal vesicle oocytes; LD: late diakinesis ooccytes; MI: metaphase I oocytes; AI/TI: anaphase I to telophase I transition oocytes; MII: metaphase II oocytes. *Statistically significant difference between control and H_2S in the same time point and the nuclear stage.

Supporting Informations S4

~~Supporting Information~~Table S4a. Effect of Na_2S on maturation of DOs after 20 hrs.

		Stage of meiotic maturation (% ± SE)					n
		GV	LD	MI	AI/TI	MII	
COCs	control	32.5±3.2^{a}	24.2±3.2^{a}	43.3±2.7^{c}	0.0±0.0^{b}	-	120
	H_2S	20.0±3.8^{b}	5.8±4.2^{b}	74.2±3.2^{a}	0.0±0.0^{b}	-	120
DOs	control	20.0±2.7^{b}	25.8±5.0^{a}	54.2±3.2^{b}	0.0±0.0^{b}	-	120
	H_2S	24.2±3.2^{b}	2.5±3.2^{b}	38.3±4.3^{c}	35.0±5.8^{a}	-	120

H_2S: 300µM Na_2S. GV: germinal vesicle; LD: late diakinesis; MI: metaphase I; AI/TI: anaphase I to telophase I transition; MII: metaphase II. ^{a,b,c}Statistically significant differences among experimental groups in the same nuclear stage – in column ($P<0.05$).

~~Supporting Information~~ Table S4b. Effect of Na_2S on maturation of DOs after 30 hrs.

		Stage of meiotic maturation (% ± SE)					n
		GV	LD	MI	AI/TI	MII	
COCs	control	0.0±0.0^{b}	-	42.5±3.2^{b}	50.8±5.0^{b}	6.7±2.7^{c}	120
	H_2S	0.0±0.0^{b}	-	15.0±1.9^{d}	69.2±3.2^{a}	15.8±4.2^{b}	120
DOs	control	5.8±3.2^{a}	-	58.3±4.3^{a}	19.2±3.2^{d}	16.7±4.7^{b}	120
	H_2S	4.2±3.2^{a}	-	26.7±2.7^{c}	39.2±5.0^{c}	30.0±5.4^{a}	120

H_2S: 300µM Na_2S. GV: germinal vesicle; LD: late diakinesis; MI: metaphase I; AI/TI: anaphase I to telophase I transition; MII: metaphase II. ^{a,b,c,d}Statistically significant differences among experimental groups in the same nuclear stage – in column ($P<0.05$).

6.2. Příloha 2: Nevoral *et al*. (2014): Cumulus cell expansion, a role in oocyte biology and its measurement: a review. Scientia Agriculturae Bohemica, *In Press*.

Cílem review bylo shrnutí problematiky kumulární expanze a její využití pro hodnocení kvality meiotického zrání a nabývání vývojové kompetence během *in vitro* kultivace kumulo-oocytárních komplexů (COCs). Práce přináší návrhy možného hodnocení kumulární expanze podle obsahu hyaluronové kyseliny (HA) produkované a zadržené v COCs. Jako vhodnou metodu měření HA lze na základě dosavadního poznání doporučit analytické metody stanovení štěpných produktů polymeru HA po enzymatické digesci.

SCIENTIA AGRICULTURAE BOHEMICA

Cumulus cell expansion, a role in oocyte biology and its measurement: a review

--Manuscript Draft--

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Corresponding Author:	Jan Nevoral, Ing. Czech University of Life Sciences Prague Prague, CZECH REPUBLIC
Corresponding Author Secondary Information:	
Corresponding Author's Institution:	Czech University of Life Sciences Prague
Corresponding Author's Secondary Institution:	
First Author:	Jan Nevoral, Ing.
First Author Secondary Information:	
Order of Authors:	Jan Nevoral, Ing.
	Matyas Orsak
	Pavel Klein
	Jaroslav Petr
	Marketa Dvorakova
	Ivona Weingartova
	Alena Vyskocilova
	Katerina Zamostna
	Tereza Krejcova
	Frantisek Jilek
Order of Authors Secondary Information:	
Abstract:	Cumulus expansion of the cumulus-oocyte complex is necessary for meiotic maturation and acquiring developmental competence. Cumulus expansion is based on extracellular matrix synthesis by cumulus cells. Hyaluronic acid is the most abundant component of this extracellular matrix. Cumulus expansion takes place during meiotic oocyte maturation under in vivo and in vitro conditions. Quantification and measurement of cumulus expansion intensity is one possible method of determining oocyte quality and optimising conditions for in vitro cultivation. Currently, subjective methods of expanded area and more exact cumulus expansion measurement by hyaluronic acid assessment are available. Among the methods of hyaluronic acid measurement is the use of radioactively labelled synthesis precursors. Alternatively, immunological and analytical methods, including Enzyme-linked Immunosorbent Assay (ELISA), Spectrophotometry and High-Performance Liquid Chromatography (HPLC) in UV light, could be utilised. The high sensitivity of these methods could provide a precise analysis of cumulus expansion without the use of radioisotopes. Therefore, the aim of this review is to summarise and compare available approaches of cumulus expansion measurement, respecting special biological features of expanded cumuli, and to suggest possible solutions for exact cumulus expansion analysis.

Cumulus cell expansion, a role in oocyte biology and its measurement: a review

J. Nevoral[1], M. Orsak[2], P. Klein[3,5], J. Petr[4], M. Dvorakova[1], I. Weingartova[1], A. Vyskocilova[1], K. Zamostna[1], T. Krejcova[1], F. Jilek[1]

[1]Department of Veterinary Sciences, Faculty of Agrobiology, Food and Natural Resources, University of Life Sciences Prague, Czech Republic

[2]Department of Chemistry, Faculty of Agrobiology, Food and Natural Resources, University of Life Sciences Prague, Czech Republic

[3]Contipro Pharma a.s., Dolni Dobrouc, Czech Republic

[4]Research Institute of Animal Production, Prague-Uhrineves, Czech Republic

[5]Current address: Biomedical Centre, Faculty of Medicine in Plzen, Charles University, Czech Republic

Abstract

Cumulus expansion of the cumulus-oocyte complex is necessary for meiotic maturation and acquiring developmental competence. Cumulus expansion is based on extracellular matrix synthesis by cumulus cells. Hyaluronic acid is the most abundant component of this extracellular matrix. Cumulus expansion takes place during meiotic oocyte maturation under *in vivo* and *in vitro* conditions. Quantification and measurement of cumulus expansion intensity is one possible method of determining oocyte quality and optimising conditions for *in vitro* cultivation. Currently, subjective methods of expanded area and more exact cumulus expansion measurement by hyaluronic acid assessment are available. Among the methods of hyaluronic acid measurement is the use of radioactively labelled synthesis precursors. Alternatively, immunological and analytical methods, including Enzyme-linked Immunosorbent Assay (ELISA), Spectrophotometry and High-Performance Liquid Chromatography (HPLC) in UV light, could be utilised. The high sensitivity of these methods could provide a precise analysis of cumulus expansion

without the use of radioisotopes. Therefore, the aim of this review is to summarise and compare available approaches of cumulus expansion measurement, respecting special biological features of expanded cumuli, and to suggest possible solutions for exact cumulus expansion analysis.

Keywords

Oocyte, cumulus-oocyte complex, cumulus expansion, hyaluronic acid, Spectrophotometry, High-Performance Liquid Chromatography.

Introduction

Reproductive biotechnologies are a dynamically developing discipline of farm animal breeding. Their progress depends on a sufficient quantity of quality oocytes useful for procedures, such as *in vitro* fertilisation, cloning by somatic cell nuclear transfer and transgenesis. The quality of oocytes is determined by the completion of cytoplasmic maturation and the acquisition of developmental competence. In these processes, cell communication between oocyte and surrounding cumulus cells, and cumulus expansion are required (Dekel et al., 1979; Eppig, 1979).

The cumulus cells with adjacent extracellular matrix constitute cumulus, an essential component of cumulus-oocyte complexes (COCs). Cumulus surrounds the oocyte during growth and consecutive meiotic maturation of the oocyte, ovulation, fertilisation and early embryonic development. During meiotic maturation of the oocyte, cumulus cells change their morphology and metabolic activity. Thus, cumulus cells significantly influence oocyte maturation and developmental competence acquisition (Sutovsky et al., 1994; Procházka et al., 2000; Qian et al., 2003; Karja, 2008; Ju and Rui, 2012; Auclair et al., 2013). In addition to cross-talk between cumulus cells and oocyte, the cumulus cells are important in the synthesis of a large quantity of extracellular matrix occurring in the enlargement of COCs. This phenomenon is known as cumulus expansion. Cumulus

expansion takes place in the follicle shortly before ovulation *in vivo,* as well as during meiotic maturation *in vitro* (Eppig, 1979; Dekel and Beers, 1980; Salustri et al., 1989; Chen et al., 1993).

Cumulus expansion is based on synthesis of glycosaminoglycan rich in hyaluronic acid (HA) into the extracellular space, where it plays a role as the structural component of expanded cumuli and signal molecule regulating oocyte maturation. A sufficient number of cumulus cell layers and adequate HA production followed by cumulus expansion are essential for successful oocyte maturation, fertilisation and early embryonic development (Chen et al., 1993; Tirone et al., 1998; Kimura et al., 2002; Han et al., 2006; Yokoo et al., 2010). Accordingly, the quality of cumuli and cumulus expansion are important markers of the COCs' quality necessary for reproductive biotechnologies (Han et al., 2006).

There are several methods of cumulus expansion measurement. Among these, non-invasive measurement by image analysis of expanded cumuli includes some disadvantages, such as the subjectivity of measurement and inability of three-dimensional structure inclusion. HA quantity measurement is the more exact invasive method of cumulus expansion quantification and measurement. Recently, HA measurement has been based on radioactive labelling of the precursor of HA synthesis (Eppig, 1980; Fagbohun and Downs, 1990; Daen et al., 1994; Nagyova et al., 1999). Alternatively, immunochemical and analytical methods are available for HA measurement (Kongtawelert and Ghosh, 1990; Volpi, 2000; Chen et al., 2005). The development of HA measurement could provide a more accurate study of cumulus expansion during oocyte maturation and qualified evaluation of the quality of COCs used for biotechnologies.

Chemistry of cumulus expansion

Cumulus cells and extracellular matrix synthesis

In the prenatal stage of ontogenesis, primordial oocytes are surrounded by one layer of follicular cells. Together with meiotic division of follicular cells and follicle growth,

differentiation of the cells to mural granulosa cells and cumulus cells of *cumulus oophorus* occurs. Cumulus cells create near surroundings of the oocyte and include *corona radiata* cells. *Corona radiata* mediates contact with the oocyte by cell connection type gap junction on *corona radiata* cell spurs permeating throughout the *zona pellucida* (Brower and Schultz, 1982; Wassarman, 1988; Morbeck et al., 1992). Individual oocytes and adjacent cumulus cells form cumulus-oocyte complexes (COCs).

Simultaneously with follicle growth, *in vivo* oocyte growth takes place inside the follicle. On pig ovaries, only follicles with diameter >3 mm include fully grown oocytes capable of reinitiating and successfully completing meiotic maturation (Marchal et al., 2002). Subsequently, with oocyte meiotic maturation within the follicle, cumulus cells synthesise structural components of extracellular matrix and the enlargement of COCs occurs. The COC extracellular matrix increases and formation during oocyte maturation has been described as mucification or cumulus expansion (Dekel et al., 1979; Eppig, 1979). Cumulus expansion occurs immediately before ovulation *in vivo*, as well as during maturation of COCs *in vitro*.

Cumulus expansion consists of the synthesis of the extracellular mass composed of proteoglycans. Proteoglycans are created by cumulus cell membrane proteins and glycosaminoglycans (GAGs). In animal tissues, hyaluronic acid (HA), chondroitin sulfate (ChS), keratan sulfate and heparan sulfate are common GAGs (Murray et al., 2003). The HA is the most abundant compound in the extracellular matrix of expanded cumuli (Chen et al., 1990; Nakayama et al., 1996; Tirone et al., 1998).

Hyaluronic acid

Hyaluronic acid is an abundant biopolymer of the GAGs family. The β-(1-4)-glucuronic acid and β-(1-3)-N-acetylglucosamine are heterodimer components of HA (Murray et al., 2003). The heterodimer is generally repeated 10 – 10 000x and the final molecular weight of HA is usually 400 kDa in bovine eye vitreous, to 1400 kDa in poultry comb (Shiedlin

et al., 2004). A large quantity of –OH groups are capable of binding many water molecules between HA chains (Murray et al., 2003). Sufficient quantity of HA synthesis precursors is a limiting factor of cumulus expansion. Their suboptimal quantity *in vitro* can create differences in cumulus expansion intensity between *in vitro* and *in vivo* conditions (Chen et al., 1990).

In many tissues, HA is extensively secreted into the extracellular matrix. Fibroblasts and other connective tissue cells are highly active in HA synthesis (Moscatelli and Rubin, 1974). Therefore, HA is ubiquitous polymer involved in joint structure and functions (Swann et al., 1974), HA is widespread in eye vitreous (Mizuno et al., 1991), umbilical cord (Lago et al., 2005) and embryonic tissues (Vabres, 2010). HA plays the role of lubricant responsible for tissue elasticity and compressibility (Swann et al., 1974). Moreover, HA participates in embryonic morphogenesis by allowing the migration of embryonic cells (Vabres, 2010). Hyaluronic acid is an important component in wound healing (Prosdocimi and Bevilacqua, 2012). The HA abundance in an organism is the cause of its release of body fluids, such as pleural fluid (Jardillier, 1972), synovial fluid (Swann et al., 1974), urine and blood (Morse and Nussbaum, 1967). The HA content in some fluids can be a useful marker of arthritis (Engstrom-Laurent and Hallgren, 1985) and liver cirrhosis (Nyberg et al., 1988). During *in vitro* cultivation of cells, HA is released into the surrounding environment such as culture medium (Johnsson et al., 1997; Chockalingam et al., 2004).

In addition, HA plays an important role in the reproductive physiology as a structural and signal molecule. HA participates in the regulation of oocyte meiotic maturation (Yokoo et al., 2010), cumulus expansion (Yokoo et al., 2003), ovulation and fertilisation of the oocyte (Chen et al., 1993), early embryonic development (Furnus et al., 2003), embryo nidation in uterine mucous membrane (Parikh et al., 2006) and foetal morphogenesis (Vabres, 2010). Moreover, the beneficial and protective effect of added HA on *in vitro* oocyte culture has been determined (Sato et al., 1987).

The HA synthesis is enzymatically catalysed by hyaluronan-synthase (HAS) isoforms. In bovine and porcine COCs, HAS2 is necessary for cumulus expansion and its expression is regulated by gonadotropins (Kimura et al., 2002; Schoenfelder and Einspanier, 2003; Nagyova et al., 2012). The mRNA HAS3 was described in matured oocytes (Kimura et al., 2002), where HA is secreted into the perivitelline space between oolema and *zona pellucida* by the matured oocyte (Ueno et al., 2009).

Cumulus expansion and meiotic maturation

Endocrine and paracrine regulation of cumulus expansion

Endocrine regulation of cumulus expansion is based on the effect of gonadotropins, such as Luteinising Hormone (LH) and Follicle Stimulating Hormone (FSH), prostaglandin E2 (PGE2) secretion and steroidogenesis including progesterone production (Dekel et al., 1979; Eppig, 1981; Schuetz and Dubin, 1981; Phillips and Dekel, 1982). These hormones influence the metabolism of granulosa cells (Wassarman, 1988), which subsequently participate in the paracrine regulation of cumulus expansion within the follicle (Motlik et al., 1998a), see Figure 1.

In paracrine regulation, the follicular fluid generated by follicular cells' secretion and substance infiltration from blood plasma during folliculogenesis, significantly contributes to cumulus expansion (Nakayama et al., 1996). Likewise, foetal bovine serum has a stimulatory effect on cumulus expansion and the chemical similarity to follicular fluid could be the explanation for this phenomenon (Eppig, 1980).

Production of the so-called Cumulus Expansion Enabling Factors (CEEFs) in granulosa and cumulus cells has been described (Motlik et al., 1998a; Prochazka et al., 1998). Whereas CEEF secretion by granulosa cells of the fully grown follicle is strictly dependent on gonadotropin control, CEEF secretion by cumulus cells does not require hormonal action and paracrine signalling is sufficient (Zhang et al., 2008). Presumably, CEEF action is linked to the inability of the complete cumulus expansion of COCs with

meiotic incompetent oocytes (Tirone et al., 1993). Although CEEF production by oocyte and cumulus cells of incompetent COCs is sufficient, the incompetent COCs lack the ability of reaction to paracrine regulation by CEEFs (Zhang et al., 2008). Many CEEFs have more exactly been defined as growth factors produced by cumulus cells as well as by oocyte.

The presence of growth factors in follicular fluid and positively modulating cumulus expansion are known as the Epidermal Growth Factor (EGF) and Insulin-like Growth Factor I (IGF-I) (Nagyova et al., 1999; Jezova et al., 2001; Nemcova et al., 2007; Nagyova, 2012). In addition to EGF and IGF-I, members of the Transforming Growth Factor β (TGFβ) superfamily, including TGFβ1, TFGβ2 and Growth Differention Factor 9 (GDF9), are required in gonadotropin-induced cumulus expansion (Vanderhyden et al., 2003; Dragovic et al., 2005). Subsequently, Drosophila Mothers Against Decapentaplegic Protein 2/3 (SMAD2/3) signal pathway is activated and the expression of important factors, such as HAS2 or PGE2 synthase, is triggered (Dragovic et al., 2007).

Another paracrine factor regulating cumulus expansion is interleukin-6 (IL-6). The IL-6 binding to its soluble receptor in cells induces genes with an impact on cumulus expansion, throughout the key factors of oocyte maturation, such as Mitogen Activated Protein Kinases (MAPK) and Akt kinase (Liu et al., 2009). The role of IL-6 in cumulus expansion is modulated by Tyrosine kinase receptor A, which is required for gonadotropin-induced follicle development (Wang et al., 2004).

Members of Hyaluronic Acid Binding Proteins (HABPs) have been indicated as substantial factors contributing to extracellular matrix formation in expanded cumuli. During cumulus expansion, Inter-α-trypsin Inhibitor (IαI) and Tumour Necrosis Factor α-induced Protein 6 (TNFAIP6) are responsible for extracellular matrix formation of expanded cumuli by the stabilisation of HA chains (Chen et al., 1992; Fulop et al., 1997; Nagyova et al., 2004; 2012). In expanded cumuli formation, the proteolytical degradation of IαI throughout the ubiquitin-proteasomal system is substantially required (Yi et al.,

2008). Cleavage of IαI to heavy chains HC1 and HC2 is necessary for HCs' binding to HA and for the stabilisation of expanded cumuli (Nagyova et al., 2004). Thus, proteasome inhibition causes complete suppression of cumulus expansion in porcine COCs (Nagyova et al., 2012).

The interaction of TNFAIP6 with HA chains is essential for the further stabilisation of expanded cumuli. This binding depends on TNFAIP6 interaction with pentraxin (PTX3), upregulated by GDF9 and produced during cumulus expansion into extracellular matrix (Varani et al., 2002; Salustri et al., 2004). Although the PTX3 does not influence HA production, it is necessary just as a further structural constituent of expanded cumuli (Salustri et al., 2004). In addition, endogenous small non-coding RNAs, called microRNAs (miRNA), of protein importance in expansion regulation are involved. The cross-talk between *Ptx3* gene and miRNA-224 as negative regulator of cumulus expansion has been described (Yao et al., 2014).

Gasotransmitters, such as nitric oxide, hydrogen sulfide and carbon monoxide (summarised by Smelcova and Tichovska, 2011) are intensively studied factors of reproductive processes, including those in meiotic maturation and cumulus expansion. It was observed that nitric oxide synthase (NOS) and nitric oxide presence as well as hydrogen sulphide in porcine COCs are important for successful oocyte maturation and cumulus expansion (Tao et al., 2005; Chmelikova et al., 2010; Amale et al., 2011; Nevoral et al., 2014). Some selected signal pathways involved in cumulus expansion regulation are shown in Figure 1.

The concentration and proportion of the regulatory factors mentioned above are responsible for various effects of different weight fractions of follicular fluid and follicular fluid from different sizes of follicle on cumulus expansion intensity (Daen et al., 1994; Dostal and Pavlok, 1996; Qian et al., 2003). The possible reason for the different action of various sizes of follicle on cumulus expansion could be the decreasing concentration of inhibitory factors in the growing follicle (Yang et al., 1993; Dostal and

Pavlok, 1996; Nandi et al., 2007). Simultaneously, the accumulation of steroid hormones (Dode and Graves, 2002) and the secretion of oocyte maturation stimulatory factors (Yoshida et al., 1992) as growth factors (Ding and Foxcroft, 1994) or amino acids (Hong et al., 2004) and electrolytes (Iwata et al., 2004) take place during follicle growth.

The role of cumulus expansion in regulation of oocyte maturation

Whereas the meiosis of dictyate fully grown oocyte in first meiotic arrest is reinitiated by hormonal stimulation *in vivo*, it is possible to evoke meiotic maturation by gonadotropins, serum proteins and growth factors also under *in vitro* conditions (Singh et al., 1997; Uhm et al., 1998). Accordingly, germinal vesicle breakdown (GVBD) occurrs and oocyte maturation is completed by achievement of the second meiotic metaphase, where meiosis is spontaneously blocked. The mature oocyte after ovulation *in vivo* or *in vitro* cultivation is predetermined for fertilisation (Motlik and Fulka, 1976; Yanagimachi, 1988; Motlik and Kubelka, 1990).

Cumulus cells are necessary for meiotic and developmental competence acquisition during oocyte growth and maturation (Chesnel et al., 1994; Qian et al., 2003; Han et al., 2006; Auclair et al., 2013). The number of cumulus cell layers, cumuli quality and cumulus expansion intensity are decisive in the success of oocyte maturation *in vitro* and required for viability of matured and fertilised oocytes (Procházka et al., 2000; Qian et al., 2003; Karja, 2008; Ju and Rui, 2012). Thus, only oocytes with an intact cover of cumulus cells in a sufficient number of layers are used for *in vitro* maturation.

Cumulus cells communicate with each other and with the oocyte by gap junctions (Gilula et al., 1978). Gap junctions facilitate the passage of molecules smaller than 1 kDa including molecules of second messengers, such as cyclic adenosine 3',5'-monophosphate (cAMP), cyclic guanosine 3',5'-monophosphate (cGMP) and Ca^{2+} ion, produced in cumulus cells. These messengers significantly inhibit key protein kinases of oocyte maturation - M-Phase Promoting Factor (MPF) and the above mentioned MAPK, and thus

GVBD and subsequent oocyte maturation (Moor et al., 1980; Dekel et al., 1988; Tornell et al., 1990; Caroll et al., 1994; Machaty et al., 1997). After the action of gonadotropin and growth factors, disruption and endocytosis of gap junction proteins and enlargement of expanding cumulus result in the prevention of cAMP/cGMP flow into oocyte (Dekel et al., 1981; Tornell et al., 1984; Chen et al., 1990; Tatemoto and Terada, 1998; Motlik et al., 1998b)(, see Figure 2. Cumulus enlargement is based in particular on HA production by cumulus cells and its accumulation in the extracellular space (Chen et al., 1990, 1993; Nakayama et al., 1996; Tirone et al., 1998). In addition to mechanical action, HA has also been described as a signal molecule and ligand.

Widespread HA receptors participating in glycoprotein creation are proteins from the above-mentioned group of HABPs. As one of the HABP members, CD44 receptor, is localised in the cytoplasmic membrane of cumulus cells and oocyte (Kimura et al., 2002; Yokoo et al., 2002). Gonadotropin-induced CD44 receptor synthesis occurs intensively during oocyte maturation (Yokoo et al., 2002; Schoenfelder and Einspanier, 2003). Subsequently, the CD44 receptor becomes active by binding HA and takes part in communication between oocyte and cumulus cells (Kimura et al., 2002). Yokoo et al. (2007) described the necessity of CD44 for meiotic maturation of porcine oocytes. Active CD44 participates in phosphorylation of gap junction proteins, cell connection closing and disruption of cAMP flow from cumulus cells to oocyte (Yokoo et al., 2010), see Figure 2. The CD44 receptor activated by HA regulates cumulus expansion and participates in auto-acceleration and amplification (Yokoo et al., 2003). However, CD44 activation is not strictly necessary for cumulus expansion (Yokoo et al., 2007). On the other hand, insufficient interaction of CD44 with HA during *in vitro* culture of COCs leads to unsuccessful oocyte maturation, fertilisation and early embryonic development (Yokoo et al., 2007). Suppression of CD44 and HA binding can be caused by glycosylation of CD44 extracellular domain by sialic acid (Bartolazzi et al., 1996). The other HA receptors are

ion channels of *Xenopus laevis* oocytes (Fraser, 1997). Besides binding HABPs, HA can participate in meiotic maturation by regulation of ion flow and membrane potential (Fraser, 1997).

Hyaluronic acid, not only the most abundant factor produced by cumuli, is involved in the regulation of oocyte maturation. Expression of some candidate biomarkers in cumulus has been selected for non-direct oocyte quality evaluation (summarised by Assou et al., 2010). Among them in cumuli, inducible Nitric Oxide Synthase (iNOS) and Heme Oxygenase 1 (HO-1), factors positively correlated with cell stress, are considered as a negative marker of developmental competence acquired during oocyte maturation (Bergandi et al., 2014). On the other hand, PTX3, Angiogenin (ANG) and Regulator of G-Protein Signalling 2 (RGS2) are highly expressed in oocytes with significantly better developmental competence (Feuerstein et al., 2012). Expression of mentioned GDF9, HAS2, SMAD2/3, COX2, PTX3 and TNFAIP6 in cumulus cells is linked with improved embryonic development through adequate cumulus expansion (Elvin et al., 1999; Pangas et al., 2002; Hazou et al., 2009), see Figure 1. There is possibly a method of utilising an expression profile of defined developmental competence biomarkers as an additional tool coupled with cumulus expansion measurement.

The importance of the oocyte in cumulus expansion regulation

The oocyte participates in the regulation of cumulus expansion (Dekel and Beers, 1980; Salustri et al., 1989). Only the fully grown oocyte disposes of the ability of adequate stimulation of cumulus expansion (Tirone et al., 1993). During oocyte maturation, the oocyte produces the above-mentioned CEEFs (Eppig et al., 1993). Oocyte activity in paracrine factor secretion, including CEEFs, decreases during meiotic maturation, especially after metaphase I achievement (Nagyova et al., 2000). The role of the oocyte in regulation of cumulus expansion differs, depending on animal species (Vanderhyden et al., 1993). While CEEFs' production by the oocyte is necessary for cumulus expansion for

mice COCs (Eppig et al., 1993), cumulus expansion of porcine COCs is independent of CEEF secretion by the oocyte (Prochazka et al., 1998). Oocyte removal by oocytectomy and *in vitro* cultivation of oocytectomised complexes have demonstrated the cumulus cell ability of CEEF production compensating for the removed oocyte (Prochazka et al., 1991; Motlik et al., 1998a). Nevertheless, cumulus expansion can be slightly inhibited in porcine oocytectomised complexes (Nakayama et al., 1996; Kimura et al., 2002). The oocyte presence influences the extracellular matrix composition of expanded cumuli: the oocyte determines the intensity of HA synthesis, but does not involve ChS quantity (Nakayama et al., 1996).

Cumulus expansion and oocyte developmental competence

Cumulus expansion and sufficient GAG synthesis by COC in the follicle are necessary for ovulation and the increased probability of enlarged COC interception by the infundibulum (Chen et al., 1993). Expanded cumuli of ovulated COCs play an important role in polyspermy prevention by selective barrier establishment. Moreover, cumulus expansion and HA synthesis are essential for sperm selection during fertilisation (Tesarik and Kopecny, 1986). Only fully capacitated sperm with complete enzymatic equipment, including hyaluronidase from sperm acrosome digesting HA polymers, are capable of passing through expanded cumuli (Dunbar et al., 1976).

Another polyspermy prevention is enlargement of the perivitelline space between oocyte and *zona pellucida* immediately after sperm penetration. The HA synthesis by oocyte and water molecule binding by HA is the reason for perivitelline space enlargement (Talbot and Dandekar, 2003). It was found that HA production by the oocyte without the need for cumulus cell synthesis causes perivitelline space enlargement. The inhibition of GAGs' synthesis in oocytes increased polyspermy incidence as response to the HA quantity decrease in the perivitelline space (Flechon et al., 2003; Ueno et al., 2009).

Cumulus expansion and HA synthesis in COCs during oocyte maturation are an important condition for the fertilisation capability and developmental competence acquisition by the oocyte (Han et al., 2006). Initial cumuli quality and size expressed by a number of cumulus cell layers are in positive correlation with the developmental competence of GV-oocytes. Although a smaller number of cumulus cell layers is not strictly limiting for oocyte maturation, it unfavourably influences early embryonic development, in comparison with a high quality cumuli cover of oocytes (Schoevers et al., 2007). It is possible to compensate for the insufficient cumuli of matured oocytes by co-culture with intact COCs during *in vitro* maturation (Luciano et al., 2005), or with disperged oviductal epithelial cells during zygote culture (Qian et al., 2005).

The early embryonic development success depends on a sufficient quantity of HA and HA synthesis precursor (Chen et al., 1990). The HA presence in culture medium for early embryos is able to improve embryo development to blastocyst stage (Kano et al., 1998; Miyoshi et al., 1999). In addition, HA binding to the CD44 receptor expressed in the early bovine embryo (Furnus et al., 2003) enhances early embryonic development (Toyokawa et al., 2005).

The cumulus cell presence significantly influences the first mitotic division of the zygote, blastocyst stage achievement and viability of embryos (Ju and Rui, 2012). Cumulus expansion intensity is positively linked to the success of embryonic development to 2-4 blatomers and blastocyst (Qian et al., 2003). Thus, cumulus expansion can be used as a developmental competence marker, useful for *in vitro* matured oocyte selection for *in vitro* fertilisation.

In addition to polyspermy suppression and early embryonic development enhancement, cumulus expansion prevents an extrauterine conception by enhanced interception by the infundibulum (Chen et al., 1993). Owing to cumulus expansion physical features, secretion of growth factors and cytokines by uterus mucous membrane important for

adhesion within the uterus, expanded cumuli of early embryos prevent a nidation in the oviduct (Parikh et al., 2006).

With respect to the importance of cumulus expansion, the measurement of cumulus expansion intensity is necessary for the evaluation of *in vitro* matured oocyte suitability for *in vitro* fertilisation, cloning and transgenesis.

Methods of cumulus expansion measurement

Visual evaluation of cumulus expansion and measurement of cumulus area

The original method of cumulus expansion measurement is subjective classification into groups by cumulus expansion intensity (Fagbohun and Downs, 1990). The result of the evaluation is a COC frequency in groups. Daen et al. (1994) described a quantifying method of cumulus expansion evaluation. On taking photographs, the area occupied by COCs is measured, using a ruler and calculated using the formula: area (mm^2) = length (mm) * width (mm) * 0.7854. Currently, photographs are able to undergo software analysis of images and the area is calculated by programmed algorithm. Both methods of classification and image analysis are non-invasive and continuous data collection during *in vitro* maturation is possible. Subjectivity and the non-recording of three-dimensional structure are disadvantages of these methods. For these reasons, classification and image analysis can not be considered as exact and relevant methods for cumulus expansion measurement.

Measurement of GAGs' content

The quantity of GAGs in expanded COCs can be used as a reliable marker of cumulus expansion. The HA, as the most abundant GAG, is suitable for detection and indication of cumulus expansion intensity. For HA measurement, the use of radioactively labelled precursors of synthesis is possible (Eppig, 1980). The [^{3}H]glucosamine (100 μCi/ml) as HA precursor, or [^{35}S]sulfate (60 μCi/ml) as ChS precursor, are used as components of

culture medium. After HA synthesis from labelled precursors by cultured COCs, cumulus cells of expanded cumuli are proteolysed and followed by HA extraction to solution. Subsequently, HA is enzymatically degraded by hyaluronidase, alternatively by chondroitinase ABC for ChS chains (Salustri et al., 1990). The HA content is deduced by radioactive signal emitted by the labelled HA precursor (Solursh, 1976). Disadvantages of the method are the inability of repeating cumulus expansion evaluation during culture of the same COCs and the manipulation with radioactive material.

Perspectives of cumulus expansion measurement

Apart from radio-labelled precursor usage, there are analytical methods for measurement of the degraded products of HA. Amongst these methods able to be considered are Spectrophotometry and High-Performance Liquid Chromatography (HPLC) in ultraviolet (UV) spectra (Rehakova et al., 1994; Volpi, 2000).

For analysis of HA degradation products, precise sample preparation consisting of expanded COCs is necessary. The preparation includes especially the washing out of culture medium components and the complete transfer of washed cumuli into tubes for analysis. In addition to COCs' preparation, aliquots of culture media are able to be removed and analysed for HA release by *in vitro* cultured COCs into the surrounding space. Subsequently, HA degradation is based on total enzymatic HA digestion by hyaluronidases. Only digesting HA chains by lyases without hydrolysis by water molecule enable double-bound creation in heterodimer of the glucuronic acid and N-acetylglucosamine absorbing the light in UV spectra (Alkrad et al., 2003; Stern and Jedrzeias, 2006). The amount of double bounds determining UV light absorbance is able to be measured by Spectrophotometry and/or HPLC (Volpi, 2000; Chen et al., 2005), summarised in Figure 3.

Adequate sample preparation and sensitivity of the afore mentioned methods would enable precise analysis of HA content retained in COCs, as well as the HA released into

the culture medium. Just the released HA analysis in culture medium could be a potent mode for non-invasive evaluation of cumulus expansion during in vitro COCs' cultivation. Currently, Spectrophotometry and HPLC methods are not routinely established for quantification and evaluation of cumulus expansion. The efficiency of HA measurement produced during cumulus expansion depends on further studies.

Conclusion

The cumulus expansion of COCs is based on GAG synthesis, especially HA (Dekel, 1979; Eppig, 1979). HA acts as a structural component and signal molecule during oocyte maturation and early embryonic development. Thus, cumulus expansion intensity is positively correlated with meiotic and developmental competence (Furnus et al., 2003; Yokoo et al., 2003; Han et al., 2006; Yokoo et al., 2010). The evaluation of cumulus expansion can be a marker of quality COCs used for *in vitro* fertilisation. HA measurement is the method of cumuli quality and cumulus expansion evaluation as a marker of oocyte maturation success and oocyte developmental competence.

There are only a few methods for the measurement of cumulus expansion intensity, either by expanded cumuli area, or indirectly by HA quantity measurement. Recent methods used for cumulus expansion evaluation contend with problems such as subjectivity and manipulation with radioactive material.

Analytical methods of HA content measurement in UV spectra, i.e. Spectrophotometry and HPLC, are prospects for real sample preparation, analysis of HA content retained in COCs and released into culture medium during cultivation and thus the sensitive determination of cumulus expansion.

Further testing experiments are necessary for the establishment of a suitable method of HA measurement as exact evaluation of cumulus expansion in routine practise.

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Corresponding Author

Ing. Jan Nevoral, Department of Veterinary Sciences, Faculty of Agrobiology, Food and Natural Resources, University of Life Sciences Prague, Kamycka 129, 165 21 Prague 6–Suchdol, Czech Republic, cell number: +420 224 382 932, e-mail: nevoral@af.czu.cz.

Figure 1. Some of signalling pathways involved in cumulus expansion. Cross-talk between cumulus cells and oocyte leads to adequate hyaluronic acid production and cumulus expansion.

Figure 2. Impact of cumulus expansion for trigger of germinal vesicle breakdown and re-initiation of oocyte maturation in pigs.

Figure 3. The possible approach to hyaluronic acid measurement and quantification of cumulus expansion (adjusted by Alkrad et al., 2003; Rehakova et al., 1994; Stern and Jedrzeias, 2006; Volpi, 2000).

Abbreviations:

ANG	angiogenin
cAMP	Cyclic Adenosine 3',5'-Monophosphate
cGMP	Cyclic Guanosine 3',5'-Monophosphate
CEEF	Cumulus Expansion Enabling Factor
ChS	Chondroitin Sulfate

904	COCs	Cumulus-Oocyte Complexes
905	COX2	Cyclooxygenase 2
906	DO	Denuded Oocyte
907	GAGs	Glycosaminoglycans
908	GDF9	Growth Differention Factor 9
909	GV	Germinal Vesicle
910	GVBD	Germinal Vesicle Breakdown
911	EGF	Epidermal Growth Factor
912	ELISA	Enzyme-linked Immunosorbent Assay
913	FSH	Follicle Stimulating Hormone
914	HA	Hyaluronic Acid
915	HABP	Hyaluronic Acid Binding Protein
916	HAS	Hyaluronan-Synthase
917	HC	HA heavy chain
918	HO-1	Heme Oxygenase 1
919	HPLC	High-Performance Liquid Chromatography
920	IGF-I	Insulin-like Growth Factor I
921	IαI	Inter-α-trypsin Inhibitor
922	IL-6	Interleukin-6
923	iNOS	inducible Nitric Oxide Synthase
924	IP3K	Phosphoinositide-3-Kinase
925	LH	Luteinising Hormone
926	MAPK	Mitogen Activated Protein Kinases
927	miRNA	microRNA
928	MPF	M-Phase/Maturation Promoting Factor
929	PG2	Prostaglandin E2
930	PTX3	Pentraxin

931	RGS2	Regulator of G-Protein Signalling 2
932	SMAD2/3	Drosophila Mothers Against Decapentaplegic Protein 2/3
933	TGFβ	Transforming Growth Factor β superfamily
934	TNFAIP6	Tumour Necrosis Factor α-induced Protein 6
935	TrkA	Tyrosine kinase receptor A

Figure 1
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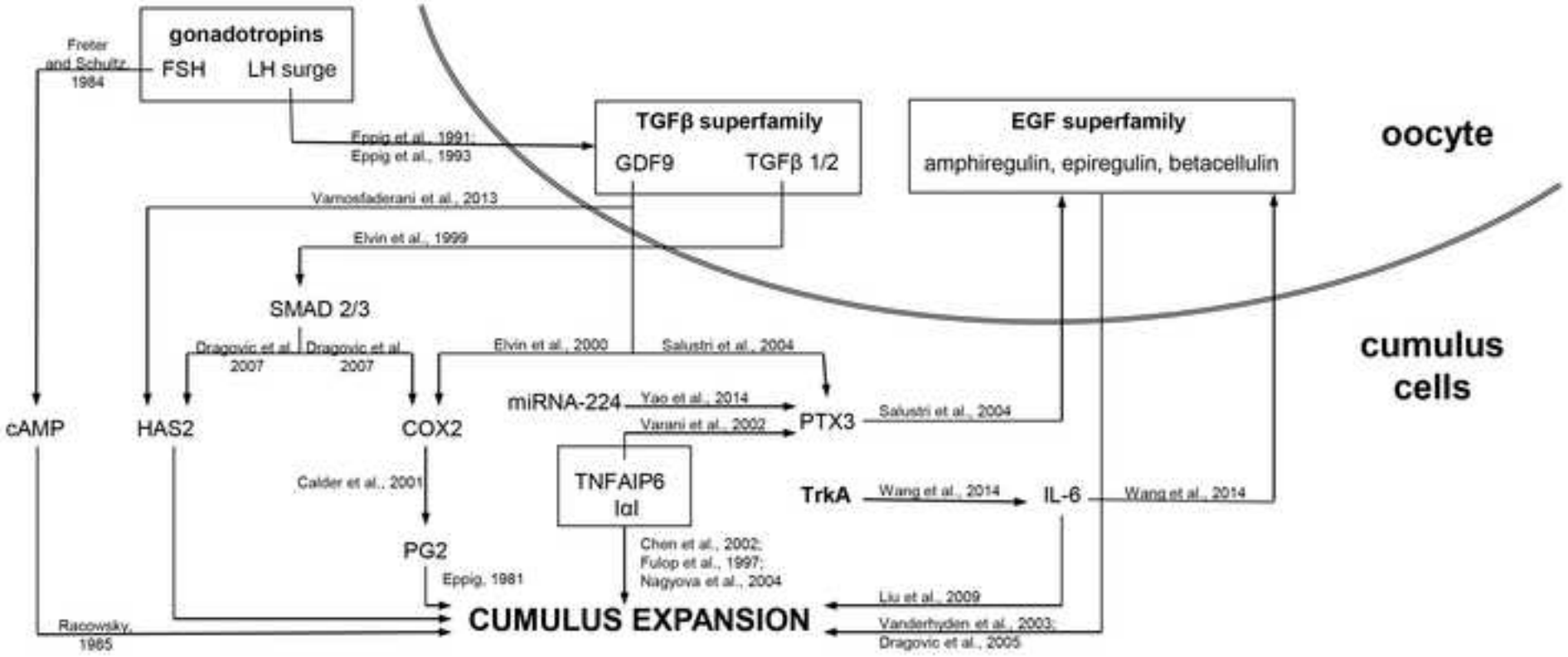

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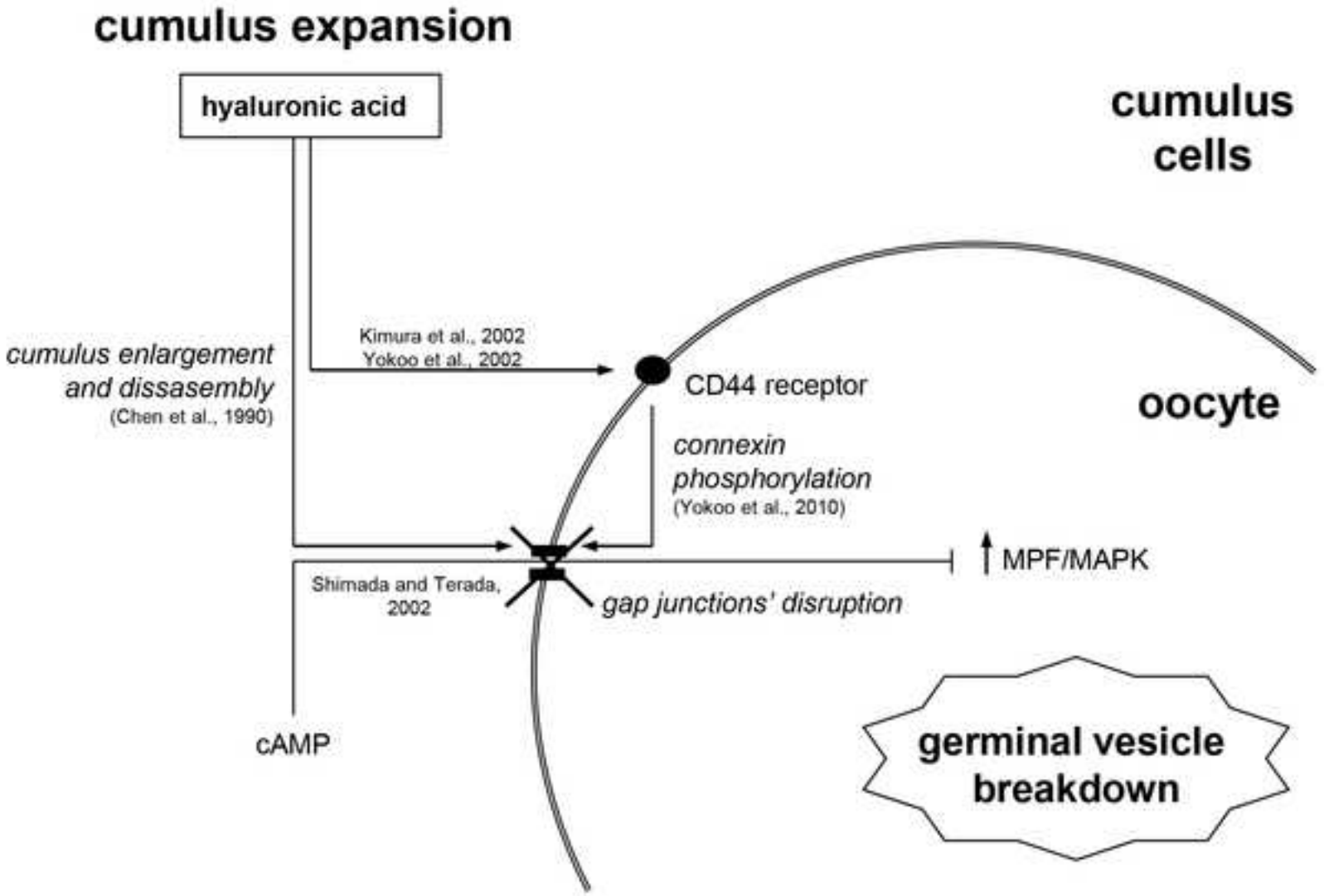

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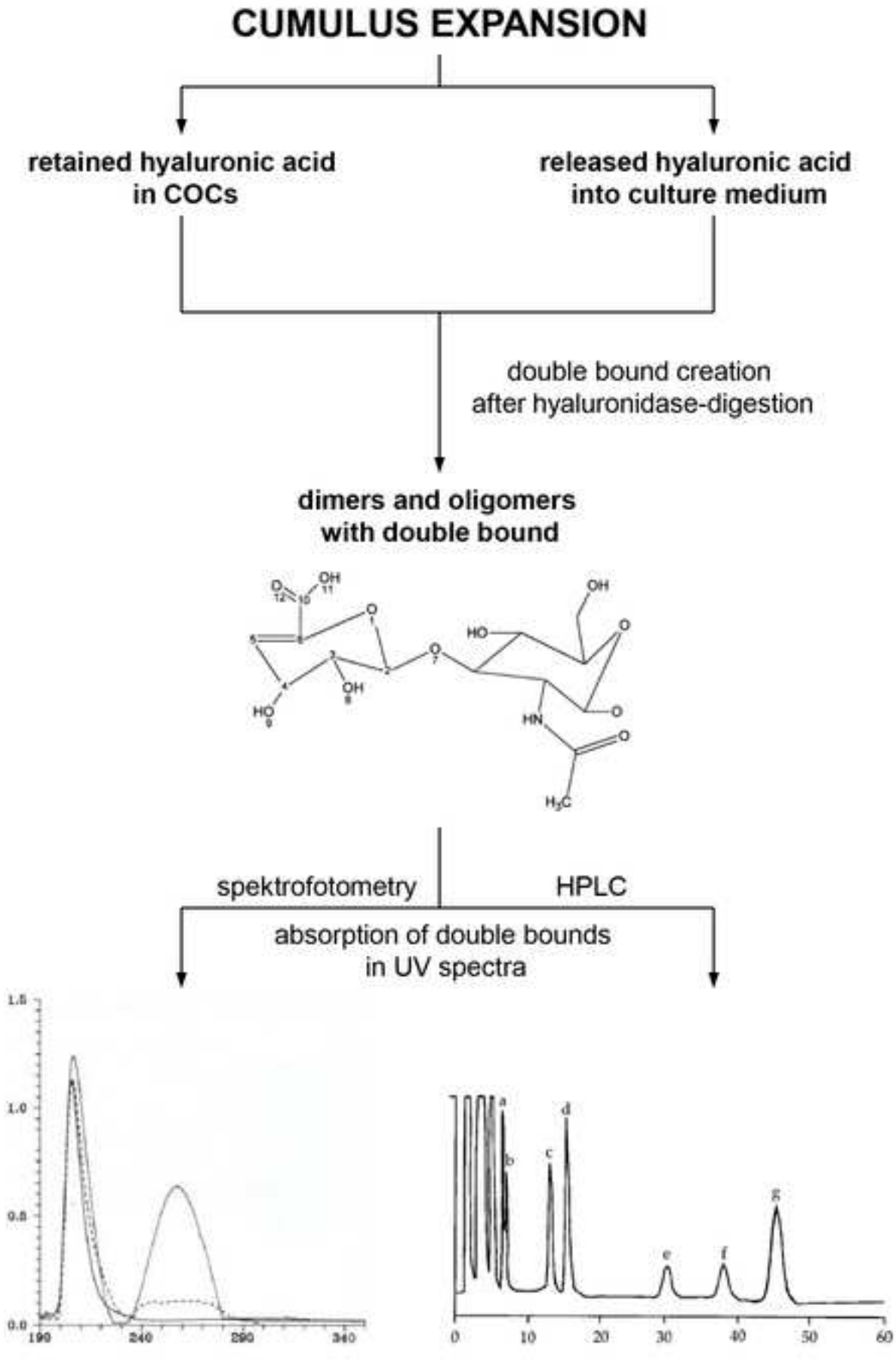

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Editorial Office
Czech University of Life Sciences Prague
Kamýcká 129, 165 21 Praha 6 – Suchdol
Tel.: +420 224 382 546,
E-mail: sab@czu.cz, web: http://sab.czu.cz

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Vážený pane kolego,

dovolte, abych Vám sdělila, že Váš příspěvek: „ Nevoral J. a kol.: Cumulus cell expansion, a role in oocyte biology and its measurement: a review“ byl přijat k uveřejnění v časopise SCIENTIA AGRICULTURAE BOHEMICA.

S pozdravem,

prof. Ing. Mgr. Markéta Sedmíková, Ph.D.

předsedkyně redakční rady

Vážený pan
Ing. Jan Nevoral
FAPPZ ČZU v Praze

6.3. Příloha 3: Sedmíková *et al*. (2013): Inhibition of c-Jun N-terminal kinase (JNK) supressess porcine oocyte ageing *in vitro*. Czech Journal of Animal Science, 58: 535-545.

Cílem práce bylo ověřit hypotézu, že c-Jun N-terminální kináza (JNK) je zapojena do procesu stárnutí prasečích oocytů během prodloužené kultivace *in vitro*. Dozrálé oocyty byly dále kultivovány 24 – 96 hod. v přítomnosti inhibitoru JNK. Následně byly stárnoucí oocyty morfologicky hodnoceny. Současně byla měřena fragmentace DNA a hodnocena exprese pro-apoptotického fatoru Bax v oocytech kultivovaných s inhibitorem JNK. Efekt inhibitoru JNK na časný embryonální vývoj byl hodnocen po partenogenetické aktivaci oocytů, vystavených prodloužené kultivaci s inhibitorem JNK. Experimenty bylo prokázáno, že JNK je zapojena do stárnutí a vyvolává nežádoucí změny oocytů, způsobující pokles jejich kvality a využití pro další postupy asistované reprodukce a reprodukčních biotechnologií. Bylo pozorováno, že inhibice JNK potlačuje tyto projevy na úrovni fragmentace DNA a časného embryonálního vývoje.

Inhibition of c-Jun N-terminal kinase (JNK) suppresses porcine oocyte ageing *in vitro*

M. Sedmíková[1], J. Petr[2], A. Dörflerová[1], J. Nevoral[1], B. Novotná[3], T. Krejčová[1], E. Chmelíková[1], L. Tůmová[1]

[1]Department of Veterinary Sciences, Czech University of Life Sciences Prague, Prague, Czech Republic
[2]Institute of Animal Science, Prague-Uhříněves, Czech Republic
[3]Department of Genetic Ecotoxicology, Institute of Experimental Medicine, Academy of Sciences of the Czech Republic, Prague, Czech Republic

ABSTRACT: Oocyte ageing is a complex of processes that occur when matured *in vitro* oocytes are, after reaching the metaphase II stage, exposed to further *in vitro* culture. Aged oocytes remaining at the metaphase II stage undergo spontaneous parthenogenetic activation, or cellular death, through apoptosis (fragmentation) or lysis. The key factor in apoptotic pathway regulation is c-Jun-N-terminal kinase (JNK), stress kinase from the mitogene-activated protein kinase (MAPK) family. To investigate the effect of JNK inhibition on porcine oocytes ageing, cleavage rate, and embryonic development after parthenogenetic activation, DNA fragmentation, and pro-apoptotic factor Bax expression, we cultured *in vitro* matured oocytes for another 1–4 days in the presence of a JNK inhibitor. The inhibition of JNK significantly protected the oocytes from fragmentation (0% of fragmented oocytes under JNK inhibition vs. 13.4% of fragmented oocytes in the control group, 2nd day of ageing) and increased the percentage of parthenogenetically activated oocytes (82 vs 57.7%, 2nd day of ageing). The embryonic development of oocytes parthenogenetically activated after 24 h of ageing was influenced by JNK inhibition as well. The percentage of oocytes at the morula stage, after seven days of cultivation, was significantly increased when oocytes aged in the presence of a JNK inhibitor (42.5%) by comparison to the percentage of oocytes exposed to ageing in an inhibitor-free medium (23.3%). DNA fragmentation was significantly suppressed by JNK inhibition from the 1st day of ageing, but the expression of pro-apoptotic factor Bax in the oocytes was not influenced. On the basis of our experiments, we can conclude that JNK inhibition suppresses apoptosis and DNA fragmentation of aged oocytes and improves their embryonic development following the parthenogenetic activation. However, to completely eliminate all ageing related processes is insufficient.

Keywords: MAPK; DNA fragmentation; apoptosis; Bax

The effectiveness of biotechnology in the types of reproduction like *in vitro* fertilization or cloning, depends on the quality of *in vitro* matured oocytes. The quality of matured oocytes decreases when they are exposed to prolonged *in vitro* cultivation. Undesirable changes in oocyte quality, called ageing, hinder the utilization of aged oocytes.

Oocyte ageing is partly due to the changes in the M-phase promoting factor (MPF) and mitogene-activated protein kinase (MAPK) activity, which are necessary to maintain the meiotic arrest in metaphase II. Furthermore, ageing is associated with abnormalities of chromosomes, defects of the meiotic spindle, mitochondrial disorders, partial exocytosis

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of cortical granules, hardening of the zona pellucida, etc. (Kikuchi et al., 2000; Miao et al., 2009; Mammucari and Rizzuto, 2010 and others). Only a small percentage of oocytes remain in metaphase II arrest, while the majority of them gradually undergo parthenogenetic activation, apoptosis or lysis (Petrová et al., 2004). Spontaneous parthenogenetic activation and release from metaphase II is related to MPF inactivation, cyclin B degradation, and a decrease in MAPK activity (Sun and Nagai, 2003; Takakura et al., 2005). Ageing decreases MAPK phosphorylation and the cleavage rate of parthenogenetically activated oocytes (Ebeling et al., 2010). Conversely, a high level of MAPK activity induces apoptosis of oocytes (Sadler et al., 2004).

A key factor in apoptotic pathway regulation is c-Jun-N-terminal kinase (JNK) from the MAPK kinase family (Dickens et al., 1997; Bagowski et al., 2003). JNK is activated in somatic cells by stress factors such as ionizing radiation, DNA damage, and responses to inflammation cytokines. Activated JNK plays a key role in the initiation of apoptosis as a stress response of the cells (Kyriakis and Avruch, 2001; Verma and Datta, 2012) and has been observed in the oocytes of *Xenopus* (Mood et al., 2004), starfish (Sadler et al. 2004), mice (Baatout et al., 2007), and swine (Petrová et al., 2009).

The downstream molecules of JNK are pro-apoptotic factors from the Bcl-2 family of proteins (Bax, Bak, Bad) (Vlahopoulos and Zoumpourlis, 2004; Chu et al., 2009; Du Pasquier et al., 2011). The activated pro-apoptotic proteins permeabilize the mitochondrial outer membrane, thereby releasing cytochrome c which, through the activation of Apaf-1, regulates the activity of efector caspase 3, and this results in DNA fragmentation (Budihardjo et al., 1999; Antignani and Youle, 2006). The involvement of factors from the Bcl-2 protein family in apoptosis and DNA fragmentation was described in cattle (Yang and Rajamahendran, 2002), mouse and human (Guillemin et al., 2009), and *Xenopus* (Du Pasquier et al., 2011) oocytes; however, there is no evidence for it in porcine oocytes.

In our previous study (Petrová et al., 2009) we determined that JNK inhibition protected the porcine oocytes from apoptosis. Conversely, it increased the percentage of spontaneously parthenogenetically activated oocytes during the entire ageing period. The aim of the present study is to examine in detail the influence of JNK inhibition on porcine oocytes ageing, particularly on DNA integrity, pro-apoptotic factor Bax expression, cleavage rate, and early embryonic development after parthenogenetic activation.

MATERIAL AND METHODS

Isolation and culture of oocytes

Pig ovaries were obtained from a local slaughterhouse from pre-pubertal gilts (mostly a crossbreed of Landrace × Large White) of local farms at an unknown stage of the estrous cycle and transported to the laboratory in a saline solution (0.9% NaCl, 39°C) within 1 h. Fully-grown oocytes were collected from follicles by aspirating those measuring 2–5 mm in diameter. Only oocytes with compact cumuli were selected for further study. Before the cultivation, the oocytes were washed three times in a maturation culture medium.

The oocytes were cultured in Petri dishes (Nunc, Roskilde, Denmark) for 48 h (39°C, 5% CO_2) with 3.0 ml of modified M199 medium Gibco BRL (Life Technologies, Carlsbad, USA) containing sodium bicarbonate (32.5mM), calcium L-lactate (2.75mM), gentamicin (0.025 mg/ml), HEPES (6.3mM), 13.5 IU eCG: 6.6 IU hCG/ml (P.G. 600) (Intervet International B.V., Boxmeer, the Netherlands), and 10% (v/v) foetal calf serum Gibco BRL (Life Technologies). For further experiment, only oocytes with a maturation rate of at least 85% were used. Matured oocytes were denuded from cumulus cells, transferred to P.G. 600 free culture medium, and subjected to culture in the presence of an active form of JNK inhibitor (1,9-parazoloanthrone, 20μM) (Calbiochem, Darmstadt, Germany) or an inactive form of JNK inhibitor (N1-methyl-1,9-pyrazoloanthrone, 20μM) (Calbiochem) for another 1–4 days.

The active and inactive forms of JNK inhibitors were dissolved in DMSO. The final DMSO concentration in the culture medium was 0.2% (v/v). The effect of DMSO (0.2%) was examined, and no significant differences were found between the groups cultured in a free medium, those cultured in the medium with DMSO, and the group cultured in the medium with the inactive form of JNK inhibitor. The data are not shown.

Evaluation of oocytes

At the end of the cultivations, the oocytes and/or embryos were mounted on slides, fixed with acetic

alcohol (1 : 3, v/v; 24 h), and stained with 1.0% (w/v) orcein. Then they were examined under a phase-contrast microscope and classified into four groups: intact oocytes (oocytes at metaphase II, anaphase II or telophase II), activated oocytes (oocytes with pronuclei or embryos), fragmented oocytes (oocytes were designated as fragmented when fragmented "vesicles" were observed under the zona pellucida), and lysed oocytes (rupture of the cytoplasmic membrane and loss of the integrity of the oocyte were the criteria for lysis) (Petrová et al., 2009).

DNA integrity assessment

DNA integrity was evaluated by the alkaline version of the Comet assay (Singh et al., 1988) modified for porcine oocytes. Briefly, the oocytes were centrifuged at 14 000 *g* for 5 min to relocate the lipids to one pole of the oocytes, and then treated with pronase (5 min, 0.1% w/v) (Sigma-Aldrich, St. Louis, USA) at 38°C to remove the zona pellucida. Subsequently, the oocytes were quickly washed in M199 culture medium, mixed with low melting point (LMP) agarose (Amresco, Solon, USA), and spread over a layer of normal melting point (NMP) agarose attached to microscopic slides SuperFrost Plus (Gerhard Menzel Glasbearbeitungswerk GmbH & Co. KG., Braunschweig, Germany) precoated with 2% agarose (for details see Novotná et al., 2010). The slides were washed with acetone (2 × 5 min) to remove the high content of lipids typical of porcine oocytes and treated in a lysing solution (2.5M NaCl, 100mM EDTA, 10mM Tris, 0.16M DMSO, 0.016mM Triton X-100, 1% lauryl sarcosinate, pH 10 – all Sigma-Aldrich) for 1.5 h.

After lysis of the oocytes, the slides were equilibrated in alkaline buffer (0.3M NaOH, 1mM EDTA, pH 13) for 40 min to allow the DNA to unwind. For distribution of DNA fragments, electrophoresis was performed in a fresh alkaline buffer (20 min, 1.2 V/cm, 300 mA). Finally, the slides were neutralized in 0.4M Tris (pH 7.5), fixed in methanol (15 min), stained with 0.005% ethidium bromide (Sigma-Aldrich) for 7 min, washed in distilled water, and dried at room temperature. To prevent artificial damage to DNA, all steps preceding the lysis were performed under yellow light. Slides rehydrated in distilled water were observed under a VANOX BHS fluorescence microscope (Olympus, Tokyo, Japan). As a relatively high number of oocytes (up to 30–40%) still retained cytoplasm (Figure 1) which prevented quantifying the percentage of fragmented DNA from the total amount of nuclear DNA, we used a qualitative evaluation of DNA damage according to Mattioli et al. (2003), i.e. visual analysis of nuclei for the presence or absence of tails of DNA fragments escaping from the nucleus (Figure 1). Images were captured with CCD 1300B camera (VDS Vosskühler GmbH, Stadtroda, Germany) and saved using Lucia G 4.81 software.

Parthenogenetic activation of oocytes

In vitro matured oocytes were denuded from cummulus cells and subjected to further culture (24 h) in the presence of an active form of JNK inhibitor or its inactive form. The oocytes were later parthenogenetically activated (calcium ionophore A23187, 25μM, 20 min and 6-dimethyl aminopurin, 2mM, 2 h (both Sigma-Aldrich) (Jílek et al., 2001) and, after being washed, cultured for another 24 h to evaluate the activation rate, 48 h to evaluate the cleavage rate, and 7 days to evaluate embryonic development. The oocyte control group was activated immediately after maturation, without a prolonged culture.

Bax localization

After cultivation, the zona pellucida of oocytes was removed by pronase (0.1% w/v) (Sigma-Aldrich – here and after unless otherwise stated) and fixed (paraformaldehyde, 2.5% w/v, 60 min). After membrane permeabilization (PBS-BSA-Triton X-100, 2 h), the oocytes were incubated with a mouse anti-Bax antibody (1 : 100, 4°C overnight), and with goat anti-mouse IgG-FITC (1 : 100, PBS-BSA-Tween 20, 60 min, RT). Nonspecific staining was determined through incubation without a primary antibody. The chromosomes were stained with Hoechst 33258 (0.5% w/v, PBS-BSA, 10 min, RT). The oocytes were mounted on slides using a SlowFade antifade kit (Invitrogen, Carlsbad, USA) and observed under a laser-scanning confocal microscope Leica TCS SPE (Leica Microsystems, Wetzlar, Germany). Signal intensity was evaluated in cytoplasm, cortical region, and nucleus (activated oocytes) using digital image analysis program NIS-Elements (Version 4.0, 2011). No significant differences were found

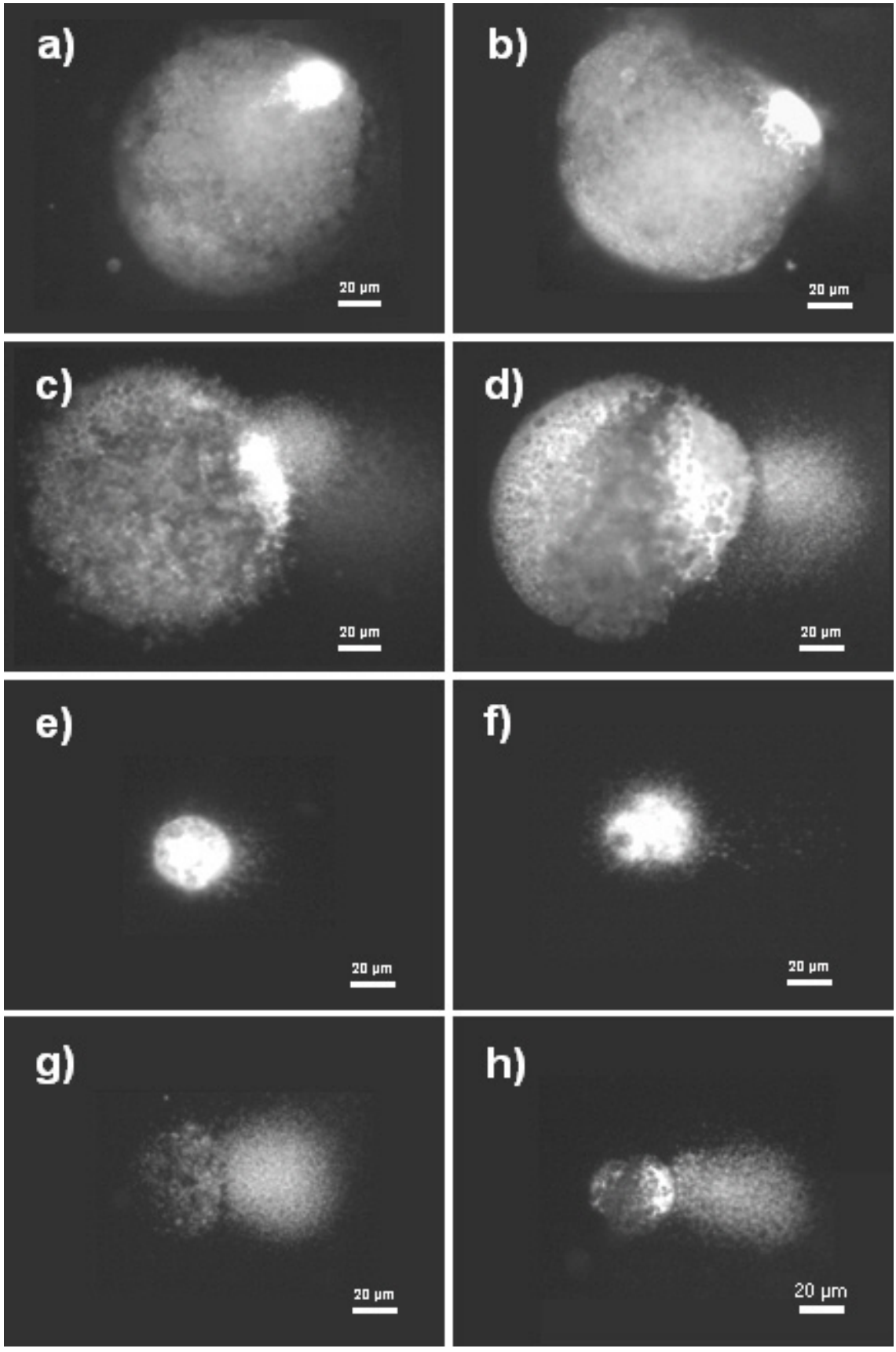

Figure 1. Porcine oocytes processed by the comet assay: (**a**–**d**) images with and (**e**–**h**) images without persisting cytoplasmic material assessed as negative (a,b,e,f) and positive (c,d,g,h) for DNA fragmentation

between signal intensity of cortical region and cytoplasm, thus the average signal intensity of each group was expressed as intensity of the whole cytoplasm and as a value relative to the average intensity of the MII oocyte group. No significant differences were found in nucleus signal intensity of activated oocytes between groups. The data are not shown. There were a minimum of 8 oocytes in each group.

Statistical analysis

Each experiment was performed at least four times. The differences among oocyte groups (days of ageing, control and experimental groups of oocytes) were evaluated by the Analysis of Variance (Scheffé's test) using the statistical software package STATISTICA (Version 8.0, 2007).

In the case of DNA integrity analysis, the data from all experiments were pooled in order to get a sufficient number of oocytes within each experimental group. Inter-group differences in the percentage of nuclei with fragmented DNA were then tested using the chi-squared test. The *P*-value of less than 0.05 was considered significant.

Experimental design

Effect of JNK inhibition on pig oocytes aged *in vitro*. *In vitro* matured oocytes were denuded of cumulus cells, placed in M199 culture medium, and cultured for another 1–4 days with a specific JNK inhibitor (1,9-pyrazoloanthrone, 20μM). The oocyte control group was cultured in the presence of an inactive form of JNK inhibitor (N1-methyl-1, 9-pyrazoloanthrone, 20μM). At the end of culture, the ratio of intact, activated, fragmented, and lytic oocytes was evaluated as described above.

Effect of JNK inhibition on the DNA fragmentation of ageing oocytes. Before the proper comet assay, the matured oocytes were cultured for 1–3 days in the presence of the active form of JNK inhibitor or its inactive form. In parallel, another group of oocytes was cultured in an inhibitor-free medium as a control.

Effect of JNK inhibition on cleavage rate and embryonic development of aged oocytes. *In vitro* matured oocytes were cultured for one more day in the presence of the active form of JNK inhibitor or its inactive form. Then, the oocytes were parthenogenetically activated and the activation rate was evaluated after 24 h. The cleavage rate was examined after additional 24 h in the culture. Embryonic development was evaluated after 7 days of culture. All assessed parameters were compared with the parameters of the oocyte control group, which was not exposed to ageing prior to parthenogenetic activation.

Effect of JNK inhibition on the subcellular localization of the pro-apoptotic factor Bax. Oocytes matured *in vitro* were cultured for another 1–3 days in the presence of the active form of

Table 1. Effect of JNK inhibition on porcine oocytes ageing

In vitro ageing (days)	Group of oocytes	Type of oocytes (% ± SEM)				
		metaphase II	activated	fragmented	lysed	*n*
1	inactive JNK inhibitor	97.7 ± 2.6^{A}	2.3 ± 2.6^{A}	0.0 ± 0.0^{A}	0.0 ± 0.0^{A}	120
	active JNK inhibitor	98.3 ± 2.6^{A}	1.7 ± 2.6^{A}	0.0 ± 0.0^{A}	0.0 ± 0.0^{A}	120
2	inactive JNK inhibitor	17.5 ± 4.4^{A}	57.7 ± 9.0^{A}	13.4 ± 3.8^{A}	11.3 ± 3.5^{A}	97
	active JNK inhibitor	14.0 ± 3.9^{A}	82.0 ± 11.2^{B}	0.0 ± 0.0^{B}	4.0 ± 2.0^{B}	100
3	inactive JNK inhibitor	4.9 ± 2.2^{A}	45.6 ± 7.6^{A}	19.4 ± 4.5^{A}	30.1 ± 5.9^{A}	103
	active JNK inhibitor	2.9 ± 1.7^{A}	79.6 ± 10.9^{B}	2.9 ± 1.7^{B}	14.6 ± 3.9^{B}	103
4	inactive JNK inhibitor	3.1 ± 1.8^{A}	38.8 ± 7.0^{A}	24.5 ± 5.3^{A}	33.7 ± 6.4^{A}	98
	active JNK inhibitor	2.0 ± 1.4^{A}	80.4 ± 11.0^{B}	1.0 ± 1.0^{B}	16.7 ± 4.2^{B}	102

oocytes were matured *in vitro* for 48 h and then further cultured for another 1–4 days in the presence of the active form of JNK inhibitor 1,9-parazoloanthrone (active JNK inhibitor) or an inactive form of JNK inhibitor N1-methyl-1,9-pyrazoloanthrone (inactive JNK inhibitor)

^{A,B}statistically significant differences between oocytes of control group (inactive JNK inhibitor) and the experimental group (active JNK inhibitor) within the same type of oocytes and day of ageing are indicated by different superscripts ($P < 0.05$)

JNK inhibitor, the inactive form of JNK inhibitor or in an inhibitor-free medium. The localization of Bax in oocytes was assayed as described above.

RESULTS

JNK inhibition showed an effect through the whole period of ageing. The percentage of parthenogenetically activated oocytes in the group cultured with an active form of JNK inhibitor was significantly higher than that in the group cultured with an inactive form of JNK inhibitor from the 2nd day of ageing. Simultaneously, the active form of JNK inhibitor suppressed fragmentation and lysis of the aged oocytes to a greater extent than did the inactive form of JNK inhibitor. The results are shown in Table 1.

In the next experiment, the effect of JNK inhibition on oocyte DNA fragmentation was studied during ageing within *in vitro* conditions. Figure 2 demonstrates that the presence of the active form of JNK inhibitor in the medium significantly decreased the percentage of oocytes with fragmented DNA when compared to oocytes ageing in the presence of the inactive form of JNK inhibitor or in a free medium (21.8% vs. 57.6% or 73.2%, respectively). However, this effect was observed only after the 1st day of prolonged culture, while no inter-group differences were detected after two and three days of prolonged culture (Figure 2).

The effect of JNK inhibition on the quality of ageing oocytes was tested by evaluating the activation rate, cleavage rate, and embryonic development of oocytes aged in the presence of the active form of JNK inhibitor. With respect to MII oocytes cultured for 1 day after maturation, the percentage of parthenogenetically activated oocytes was

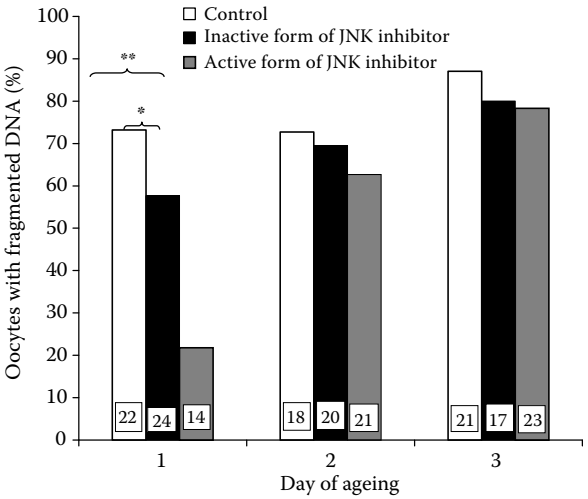

Figure 2. Effect of JNK inhibition on DNA fragmentation (%) in porcine oocytes ageing *in vitro.* Oocytes were matured *in vitro* for 48 h and then further cultured for 1–3 days, either in the pure medium (control) in the presence of an active form of JNK inhibitor (1,9-parazoloanthrone) or of an inactive form of JNK inhibitor (N1-methyl-1,9-pyrazoloanthrone)

The number in each column represents the total analyzed oocytes count within a given group; $^{*}P < 0.05$, $^{**}P < 0.01$

Table 2. Effect of JNK inhibition on porcine oocytes ageing *in vitro* and their subsequent activation, cleavage, and embryonic development (n = 120 per each group)

Stage of development/duration of culture after activation	Group of oocytes (%)		
	control (without ageing)	inactive JNK inhibitor (24 h of ageing)	active JNK inhibitor (24 h of ageing)
Activation/24 h	91.7^{A}	79.2^{B}	88.3^{A,B}
Cleavage/48 h	90.0^{A}	43.3^{B}	51.6^{B}
Stage of morula/7 days	16.7^{A}	23.3	42.5^{B}
Stage of blastocyst/7 days	30.0^{A}	0.0^{B}	0.0^{B}

oocytes were matured *in vitro* for 48 h to the MII stage and then cultured for further 24 h in the presence of an active form of JNK inhibitor 1,9-parazoloanthrone (active JNK inhibitor) or an inactive form of JNK inhibitor N1-methyl-1,9-pyrazoloanthrone (inactive JNK inhibitor). Both groups of oocytes were then parthenogenetically activated by calcium ionophore and 6-DMAP. Control group (control) was activated without ageing

^{A,B}statistically significant differences between oocytes of control groups (control and inactive JNK inhibitor) and the experimental group (active JNK inhibitor) within the same time of cultivation (in the same row) are indicated by different superscripts ($P < 0.05$)

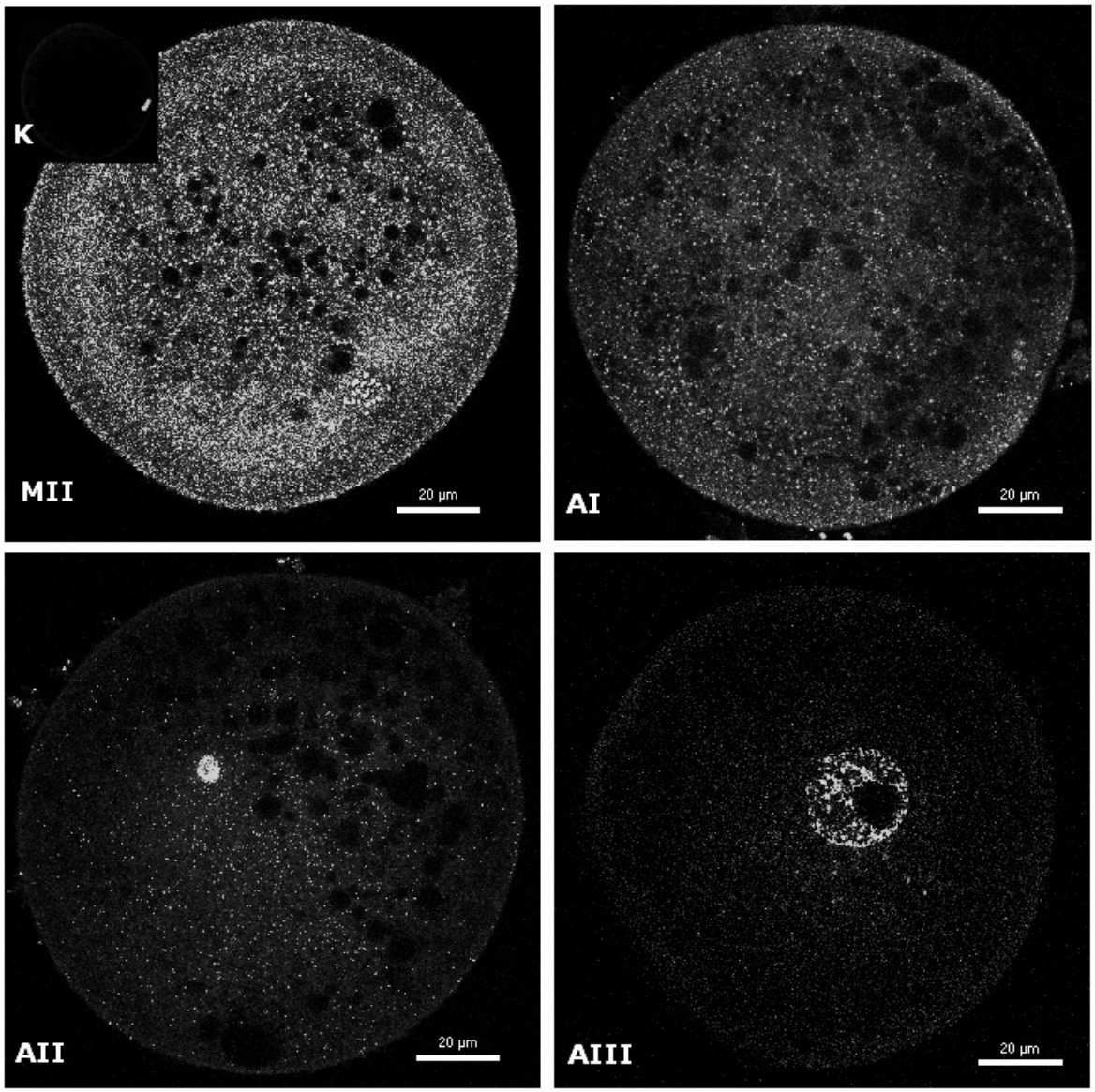

Figure 3. Subcellular localization of Bax in aged pig oocytes

K = matured MII oocyte (control oocyte exposed only to the second antibody), MII = matured MII oocyte, AI = aged oocyte (1 day after ageing), AII = aged oocyte (2 days of ageing), AIII = aged oocyte (3 days of ageing)
oocytes were stained with mouse anti-Bax and anti-mouse IgG-FITC (protein) and Hoechst 33258 (DNA)

lower in comparison with oocytes cultured for a prolonged period (Table 2). The percentage of activated oocytes in the group cultured during ageing in the presence of an inactive form of JNK inhibitor was significantly lower than that of the control group of oocytes, which were not too exposed to prolonged culture. However, the percentage of parthenogenetically activated oocytes in the group aged in the presence of the active form of JNK inhibitor was not significantly different from the percentage of activated oocytes in the control group, nor was it significantly different from the group aged in the presence of an inactive form of JNK inhibitor.

The cleavage rate of the oocytes after 24 h of ageing was significantly lower than that of the control group without ageing (51.6% – active form of JNK inhibitor or 43.3% – inactive form of JNK inhibitor vs. 90% – control). Compared to the inactive form, the active form of JNK inhibitor in culture medium did not increase the oocyte cleavage rate during ageing (43.3 vs. 51.6%); however, it significantly increased the percentage of the oocytes that reached the morula stage after

Table 3. Effect of JNK inhibition on subcellular localization of the pro-apoptotic factor Bax in ageing porcine oocytes

In vitro ageing (days)	Group of oocytes ($\bar{x}$ ± SEM)					
	control	*n*	inactive JNK inhibitor	*n*	active JNK inhibitor	*n*
0	$1.0 \pm 0.1^{A,a}$	10	–	–	–	–
1	$1.4 \pm 0.2^{A,b}$	11	$1.4 \pm 0.2^{A,b}$	9	$1.4 \pm 0.1^{A,b}$	13
2	$0.7 \pm 0.1^{A,c}$	9	$0.9 \pm 0.2^{A,c}$	9	$1.1 \pm 0.1^{A,c}$	10
3	$0.4 \pm 0.0^{A,c}$	8	$0.3 \pm 0.1^{A,d}$	9	$0.3 \pm 0.1^{A,d}$	9

oocytes were matured *in vitro* for 48 h to the MII stage (0 days of ageing) and then further cultured for another 1–3 days in the presence of an active form of inhibitor JNK 1,9-parazoloanthrone (active JNK inhibitor) or an inactive form of JNK inhibitor N1-methyl-1,9-pyrazoloanthrone (inactive JNK inhibitor) or in the inhibitor free culture medium (control). The signal intensity is expressed as a mean relative intensity based on MII oocytes (control, 0 days of ageing)

[A]statistically significant differences between oocytes of control groups (control and inactive JNK inhibitor) and the experimental group (active JNK inhibitor) within the same day of ageing ($P < 0.05$)

[a–d]statistically significant differences between oocytes of the same group during ageing are indicated by different superscripts ($P < 0.05$)

7 days of cultivation (Table 2). Interestingly, only control group oocytes that were not exposed to prolonged culture reached the blastocyst stage.

The intracellular expression of the pro-apoptotic factor Bax, influenced by the active form of JNK inhibition, expressed as mean fluorescence signal intensity in the whole cytoplasm of oocytes, was evaluated by picture analysis (Table 3, Figure 3). The highest mean intensity in the oocytes was found after the 1st day of ageing. The mean signal intensity gradually diminished during the following 2 days of ageing. However, the presence of the active form of JNK inhibitor in the culture medium did not influence the expression of the pro-apoptotic Bax factor.

DISCUSSION

In our study, we observed the dynamics of the effect of JNK inhibition on the morphological manifestation of ageing of porcine oocytes under *in vitro* conditions. The inhibition of JNK suppresses the apoptosis and lysis of oocytes and the manifestation of the effect is statistically significant from the 2nd day of oocyte ageing and persists in oocytes which were subjected to ageing for the duration of four days as well. The inhibition of JNK in our experiments significantly suppressed the fragmentation of DNA in porcine oocytes, but only during the 1st day of ageing. From the 2nd day of ageing, the differences were not statistically significant.

JNK is one of the main regulatory factors of apoptosis in somatic cells (Dhanasekaran and Reddy, 2008; Plotnikov et al., 2011). Caspase-3 and caspase-9 belong among the downstream molecules and their activation leads to DNA fragmentation (Chen et al., 2003). The results of our experiments suggest that JNK-regulated signalling is also involved in the control of apoptosis in porcine oocytes.The JNK inhibition did not manage to completely suppress the DNA fragmentation. JNK-induced signalling is probably not the only one involved in oocyte apoptosis induction.

One of the activating stimuli of JNK is oxidation stress and the increase of oxygen-free radicals (ROS) level (Levkovitz et al., 2005; Antignani and Youle, 2006). Some authors consider oxidation stress to be one of the main causes of ageing (Cui et al., 2011) and the ROS level continously increases during the ageing process (Miao et al., 2009). ROS induce apoptosis not only by activating JNK and the subsequent release of cytochrome c from mitochondria, but also through endoplasmic reticulum (ER) (He et al., 2008) which is closely related to maintaining intracellular calcium homeostasis (Petr et al., 2001). Increased ROS level affects calcium signalling and leads to the increase of cytoplasmic concentration of calcium cations (Takahashi et al., 2003).

During our experiments, the ratio of spontaneously parthenogenetically activated oocytes increased in the group of oocytes ageing in the presence of the active form of JNK inhibitor. For

the MII block output, the increase of cytoplasmic concentration of calcium cations is crucial (Wassarman, 1988; Wang et al., 1999; Sedmíková et al., 2003, 2006). Therefore, the increase of parthenogenetically activated oocytes ratio may be caused by the fact that blocking the apoptotic signalling controlled by JNK induces spontaneous parthenogenetic activation of the oocytes by increasing the levels of calcium cations.

JNK signalling leads to DNA fragmentation through pro-apoptotic factors Bax and Bad which, by mitochondrial membrane permeabilization, releases cytochrome c (Vlahopoulos and Zoumpourlis, 2004; Levkovitz et al., 2005; Antignani and Youle, 2006; Chu et al., 2009). By means of Bax (but not Bad) it takes part in controlling the JNK apoptosis in *Xenopus* oocytes. In the oocytes the JNK-induced apoptosis is manifested by overexpression of Bax protein (Du Pasquier et al., 2011). We found no differences in the expression of Bax in porcine oocytes dependent on an active JNK signalling pathway in our experiments.

It seems that Bax does not take part in apoptosis induction in porcine oocytes. It is also possible that in porcine oocytes, in contrast to *Xenopus* oocytes, the main downstream molecule of JNK is the pro-apoptotic factor Bad, similarly to some somatic cells (Bhakar et al., 2003). The presence of the active form of JNK inhibitor during porcine oocytes ageing improves their development competence, even though the percentage of parthenogenetically activated oocytes in the cleavage stage is lower. The ratio of parthenogenetically activated oocytes among ageing oocytes decreases proportionally to the duration of ageing. The ability of embryonic development in activated oocytes also decreases (Ebeling et al., 2010) and JNK apparently no longer has an influence.

The lower ratio of parthenogenetically activated oocytes may also be influenced by the function of ER affected by oxidation stress. Suppression of ER stress improves the embryonic development of porcine oocytes, due to a blockage of apoptosis induced by ER stress (Zhang et al., 2012). Greater development competency, when exposed to the effects of a JNK inhibitor, is apparently influenced by the suppression of DNA fragmentation by means of caspase activation. The course of early embryonic development is closely related to apoptosis (Antunes et al., 2010) and the effect of caspase-3 inhibitor on a higher ratio of blastocysts in parthenogenetically activated embryos has been described in porcine (Coutinho et al., 2011). The inhibition of JNK seems to be a possible way of preventing detrimental processes accompanying the ageing of mammalian oocytes, thus it could be used for improving the quality of *in vitro* matured oocytes in biotechnologies.

CONCLUSION

The results of our experiments show that JNK inhibition suppresses apoptosis manifestation in ageing porcine oocytes and improves their early embryonic development following the parthenogenetic activation. However, sole JNK inhibition does not manage to eliminate all the processes connected with oocyte ageing during prolonged cultivation under *in vitro* conditions.

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Corresponding Author

Prof. Mgr. Ing. Markéta Sedmíková, Ph.D., Czech University of Life Sciences Prague, Department of Veterinary Sciences, Kamýcká 129, 165 21 Prague 6-Suchdol, Czech Republic
Tel.: +420 224 382 933, fax +420 234 381 841, e-mail: sedmikova@af.czu.cz

6.4. Příloha 4: Nevoral *et al*. (2013): The role of nitric oxide synthase isoforms in aged porcine oocytes. Czech Journal of Animal Science, 58: 453-459.

Cílem práce bylo lokalizovat izoformy NO syntázy (NOS) ve stárnoucích oocytech prasete a hodnotit vliv inhibice NOS. Pro hodnocení byly použity oocyty dozrálé v podmínkách *in vitro*, které byly následně inkubovány s inhibitory NOS, nespecifickým inhibitorem L-NAME nebo specifickým inhibitorem aminoguanidinem po dobu 72 hodin. Bylo zjištěno, že všechny izoformy NOS, iNOS, eNOS a nNOS, jsou přítomny ve stárnoucích oocytech prasete a jejich množství v nich klesá již po 24 hod. prodloužené kultivace. Současně bylo zjištěno, že po 72 hod. prodloužené kultivace byla inhibicí NOS signifikantně potlačena buněčná smrt oocytů, zahrnující apoptózu a lýzu, přestože množství NOS v těchto oocytech prudce klesla. Na základě těchto výsledků lze předpokládat, že fyziologická produkce NO sehrává úlohu v procesech stárnutí oocytů, spontánní partenogenetické aktivaci a programované buněčné smrti.

The role of nitric oxide synthase isoforms in aged porcine oocytes

J. Nevoral[1], T. Krejčová[1], J. Petr[2], P. Melicharová[1], A. Vyskočilová[1], M. Dvořáková[1], I. Weingartová[1], E. Chmelíková[1], L. Tůmová[1], K. Hošková[1], V. Kučerová-Chrpová[1], M. Sedmíková[1]

[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

ABSTRACT: In the sphere of reproductive biotechnologies, the demand for sufficient numbers of high-quality oocytes is still increasing. In some cases, this obstacle is overcome by *in vitro* prolonged cultivation. However, a prolonged oocyte culture is accompanied by changes called ageing. Ageing is manifested by spontaneous parthenogenetic activation, programmed cell death or lysis. Various substances, such as caffeine or dithiothreitol, have been tested for ageing suppression. In this respect, research into gasotransmitters (hydrogen sulphide, carbon monoxide, and nitric oxide) has currently been intensified. The objectives of the present study were to localize nitric oxide synthases (NOS) and to evaluate NOS inhibition of aged porcine oocytes. We demonstrated the presence of NOS isoforms in oocyte cultivation prolonged by 24, 48, and 72 h. After 72 h of prolonged cultivation, NOS inhibition by the non-specific inhibitor L-NAME or the specific inhibitor aminoguanidine caused suppression both of programmed cell death and lysis. Although NOS amount rapidly decreased after the 72-h cultivation, changes induced by NOS inhibition were statistically significant. We can presume that NOS play an important physiological role in porcine oocyte ageing.

Keywords: nitric oxide; L-NAME; aminoguanidine; oocyte ageing; pig

Fully grown oocytes undergo meiotic resumption during mammal oogenesis. Meiotic maturation starts with germinal vesicle breakdown, passing through metaphase I, anaphase I, and telophase I to metaphase II (Motlik and Fulka, 1986; Wassarman, 1988). After reaching metaphase II (MII), oocytes are spontaneously arrested until fertilization and activation (Yanagimachi, 1988). Reproductive biotechnologies use MII oocytes matured *in vitro* conditions for *in vitro* fertilization, transgenesis or nuclear transfer cloning.

Sufficient numbers of high-quality MII oocytes are necessary for successful use in biotechnology. To meet this requirement, MII oocytes exposed to prolonged cultivation are frequently used. During prolonged cultivation, oocytes exhibit a series of complex changes called ageing (Petrová et al., 2004, 2009). Ageing is based on biochemical changes of key factor activities as well as on damage to the ultrastructural cytoskeleton and various organels (Kikuchi et al., 2000; Suzuki et al., 2002). These changes result in the following morphological manifestations: spontaneous parthenogenetic activation, programmed cell death (apoptosis, fragmentation), and lysis (Petrová et al., 2004; Miao et al., 2009).

Research into biochemical and structural changes during oocyte prolonged cultivation in order to postpone the onset of undesirable changes in aged oocytes is needed. Various substances were tested

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to suppress the ageing, such as dithiotreitol (Tarín et al., 1998), caffeine (Kikuchi et al., 2000), growth factors (Petrová et al., 2005), or trichostatin (Ješeta et al., 2008). Recently, the role of gasotransmitters (hydrogen sulphide (H_2S), carbon monoxide (CO), and nitric oxide (NO)) in the reproductive system has been observed (e.g. Šmelcová and Tichovská, 2011). The gastrotransmitters are expected to affect the ageing process.

As concerns NO, its function as a cell messenger has been examined in the cardiovascular system (Gómez-Fernández et al., 2004), nerve tissues (Muramatsu et al., 2000), and in reproductive processes, particularly in folliculogenesis, meiotic maturation, and ovulation (Jablonka-Shariff and Olson, 1998, 2000). NO is necessary for germinal vesicle breakdown and metaphase I to metaphase II transition (Chmelíková et al., 2010). Moreover, NOS inhibition supresses cumulus expansion of sheep oocytes (Amale et al., 2011). The role of NO in oocyte activation and early embryonic development of fertilized or parthenogenetically activated oocytes was also studied (Goud et al., 2008; Krejčová et al., 2009). Although not essential for mouse oocyte fertilization (Hyslop et al., 2001), NO is able to activate the oocytes of pigs and amphibians (Petr et al., 2005, 2010; Jeseta et al., 2012), presumably throughout cGMP and PKG signalling pathways (Petr et al., 2006).

In vivo NO production is carried out by nitric oxide synthase (NOS), an enzyme catalyzing L-arginine conversion to citrulin and NO (Kwon et al., 1990; Lamas et al., 1992). NOS exists in three isoforms: endothelial (eNOS), neuronal (nNOS), and inducible (iNOS). Whereas eNOS and nNOS are Ca^{2+}-dependent and produce a small amount of NO in a short time (Bredt et al., 1991; Lamas et al., 1992), iNOS is Ca^{2+}-independent and steadily generates 100 to 1000 times higher levels of NO for hours at a time (Nathan, 1992; Xie et al., 1992). All of the three NOS isoforms and NOS mRNA were detected in mammal oocytes and cumulus cells (Van Voorhis et al., 1995; Hattori et al., 2000).

The role of NOS and NO in physiological processes has been studied by various methods including genetic knock-out (Jablonka-Shariff and Olson, 1998) and NOS inhibitor use (Goud et al., 2008). For NOS inhibition in oocytes, a non-specific inhibitor, N^{ω}–nitro-L-arginine methylester (L-NAME) (Abavisani et al., 2011) or a specific iNOS inhibitor (aminoguanidine) were used (Chmelíková et al., 2010).

The involvement of NO in programmed cell death of neurons was demonstrated by Kang et al. (2004). Moreover, the influence of NOS was described in early embryonic development (Saugandhika et al., 2010). The NOS isoforms effects could be expected in the case of prolonged mammal oocyte cultivation, too.

The present study has two objectives: (1) to demonstrate the presence of NOS isoforms in aged porcine oocytes and describe their dynamics during prolonged cultivation and (2) to evaluate NOS inhibition influence on ageing processes after 72 h of prolonged cultivation.

MATERIAL AND METHODS

Isolation and cultivation of porcine oocytes

Porcine ovaries were obtained from non-cycling gilts at a slaughterhouse. Ovaries were transported to the laboratory in a saline solution (0.9% NaCl) at 39°C. Oocytes were collected from ovarian follicles (2–5 mm) with a 20-gauge aspirating needle. Only fully grown oocytes with intact cytoplasm and compact cumuli were used in further experiments.

The oocytes were maturating in a modified M199 medium (Sigma-Aldrich, St. Louis, USA) containing sodium bicarbonate (32.5mM), calcium L-lactate (2.75mM), gentamicin (0.025 mg/ml), HEPES (6.3mM), 13.5 IU eCG: 6.6 IU hCG/ml (P.G. 600) (Intervet International B.V., Boxmeer, the Netherlands) and 10% (v/v) foetal calf serum (GibcoBRL) (Life Technologies, Darmstadt, Germany). The oocytes were getting matured in 3.5 cm Petri dishes (Nunc, Roskilde, Denmark) containing 3.0 ml of culture medium at 39°C in a mixture of 5.0% CO_2 in air.

After *in vitro* maturation, the cumuli were removed by pipetting through a narrow glass capillary. The denuded oocytes were exposed to prolonged cultivation for 24, 48, and 72 h, and under the same conditions as by the oocyte maturation.

NOS subcellular localization in oocytes after prolonged cultivation

The zonae pellucidae of oocytes cultured for 24, 48 or 72 h were removed by a 0.1% (w/v) pronase treatment (Sigma-Aldrich). The oocytes were then washed three times in 0.01M phosphate-buffered saline (PBS) with 0.1% bovine serum albumin

(BSA), and fixed in 2.5% (w/v) paraformaldehyde in PBS for 1 h. After treatment in PBS – 0.5% BSA – 0.5% Triton X-100 (Sigma-Aldrich) for 2 h, the oocytes were incubated overnight at 4°C with mouse polyclonal anti-eNOS, anti-nNOS or anti-iNOS (1 : 100; Sigma-Aldrich) diluted in PBS – 0.1% BSA – 0.1% Tween 20 (Sigma-Aldrich). After being washed in PBS – 0.1% Tween 20, the oocytes were incubated at room temperature for 60 min with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (1 : 100; Sigma-Aldrich). Chromatin was stained with 0.5% (w/v) Hoechst 33258 (Sigma-Aldrich). The oocytes were mounted on slides with a SlowFade antifade kit (Invitrogen, Carlsbad, USA) used according to protocol. Images were obtained using a laser scanning confocal microscope Leica TCP SPE (Leica Microsystems, Wetzlar, Germany), and signal intensity was measured through imaging analysis by NIS Elements 3.00 (Laboratory Imaging s.r.o., Prague, Czech Republic). Measured values were related to MII oocytes and expressed relatively.

NOS inhibition during prolonged oocyte cultivation

In vitro matured and denuded oocytes were for 24, 48 and 72 h exposed to prolonged cultivation with a non-specific inhibitor, namely N^{ω} –nitro-L-arginine methylester L-NAME (2.5mM, 5mM, 7.5mM, 10mM; Sigma-Aldrich), or with a specific iNOS inhibitor, namely aminoguanidine (2.5mM, 5mM, 7.5mM, 10mM; Sigma-Aldrich). The oocytes were later mounted on slides and fixed in acetic alcohol (1 : 3, v/v) for 48 h. Morphological changes of oocytes were observed after staining with 1.0% orcein under a phase contrast light microscope. The oocites were classified into four groups: intact MII oocytes, parthenogenetically activated, fragmented (apoptotic), and lytic oocytes.

Statistical analysis

All the experiments were repeated four times and subjected to statistical analysis using SAS software (Statistical Analysis System, Version 9.0, 2008). The differences of signal intensity and percentages of morphological stages between the experimental groups and control were evaluated by the Analysis of Variance (Sheffé's test) and the non-parametric *F*-test, respectively. *P*-value of less than 0.05 was considered statistically significant.

RESULTS

Subcellular localization of NOS isoforms in aged oocytes

The aim of the experiment was to localize and quantify NOS isoforms in porcine oocytes during prolonged *in vitro* cultivation.

All three NOS isoforms were localized in matured and aged oocytes after 24, 48, and 72 h of prolonged cultivation (Figure 1). There were subcellular localization differences between the individual isoforms. Endothelial NOS frequently occurred in small cytoplasmic foci in aged oocytes. Neuronal NOS was observed close by the cytoplasmic membrane and parthenote pronucleus. Inducible NOS was detected especially near the cytoplasmic membrane.

Isoform dynamics during prolonged cultivation was analyzed. After 24 h of prolonged cultivation the signal intensity of nNOS and iNOS decreased by 63 and 88%, respectively, while after 48 and 72 h the signal intensity was invariable. In the case of eNOS, the signal intensity decrease after 24 h was not statistically significant; its dramatic decrease came after 72 h of prolonged cultivation (Table 1).

NOS inhibition during oocytes prolonged cultivation

The aim of the second experiment was to evaluate the influence of NOS inhibitors on oocytes aged for 72 h. The non-selective inhibitor L-NAME

Table 1. Signal intensity of nitric oxide synthases (NOS) in porcine oocytes during prolonged cultivation

	MII	24 h	48 h	72 h
eNOS	1 ± 0.32^{a}	0.79 ± 0.19^{a}	0.76 ± 0.13^{a}	0.50 ± 0.21^{b}
nNOS	1 ± 0.17^{a}	0.37 ± 0.04^{b}	0.36 ± 0.02^{b}	0.34 ± 0.03^{b}
iNOS	1 ± 0.22^{a}	0.12 ± 0.04^{b}	0.15 ± 0.04^{b}	0.10 ± 0.02^{b}

eNOS = endothelial isoform, nNOS = neuronal isoform, iNOS = inducible isoform

signal intensity is related to metaphase II (MII) oocytes; statistically significant differences during prolonged cultivation for the same isoform (i.e. within the rows) are indicated by different superscripts ($P < 0.05$)

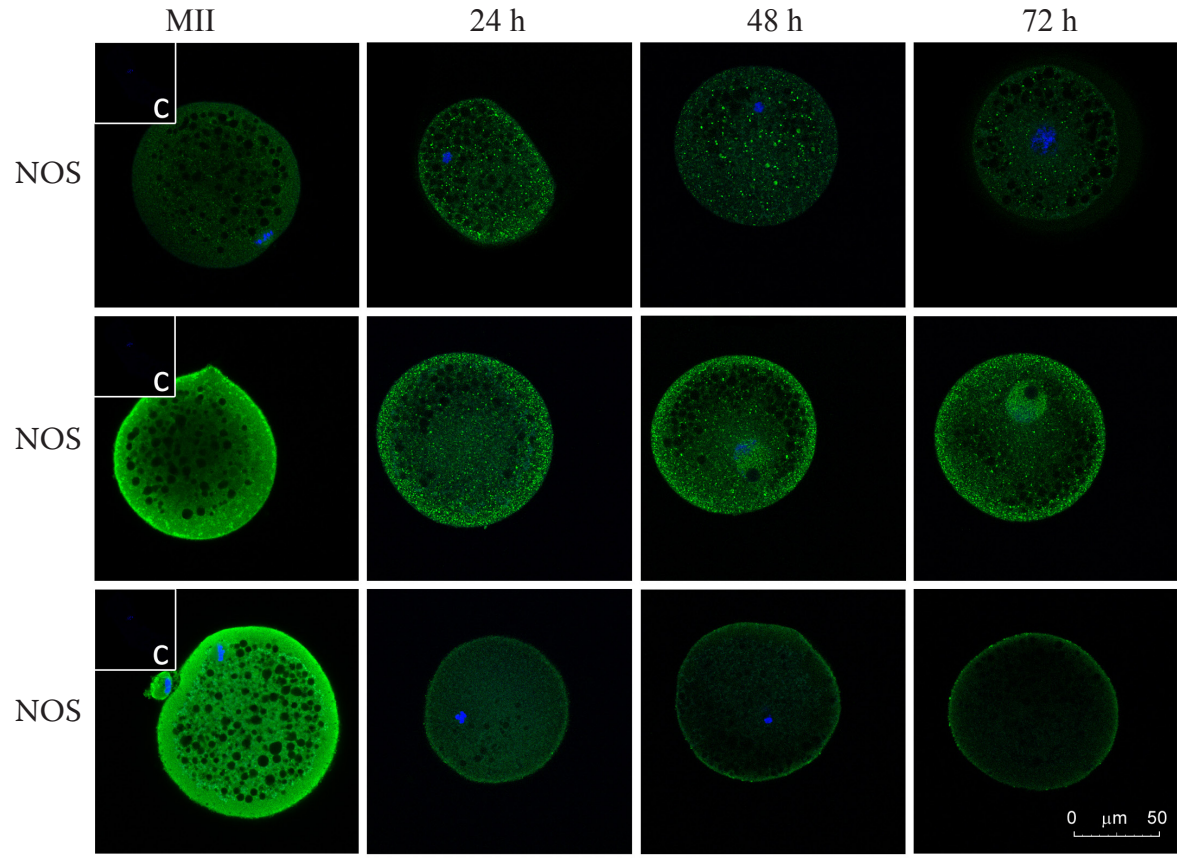

Figure 1. Subcellular localization and distribution of nitric oxide synthase (NOS) isoforms in MII and aged porcine oocytes. Oocytes were stained with mouse anti-eNOS, anti-nNOS or anti-iNOS and anti-mouse IgG-FITC. DNA was stained with Hoechst 33258. Magnification 400×

MII = matured MII oocytes without prolonged cultivation, 24 h, 48 h, 72 h = hours of prolonged cultivation, C = control oocytes exposed only to anti-mouse IgG

and selective inhibitor aminoguanidin were used. Morphological changes in oocytes ageing for 72 h under NOS inhibition were evaluated.

Both inhibitors suppressed cell death (apoptosis and lysis) in a dose-dependent manner. After 72 h of prolonged cultivation in 10mM L-NAME and 10mM aminoguanidin, only 1 and 8% of aged oocytes, respectively, succumbed to cell death. On the contrary, more oocytes remained intact or parthenogenetically activated (Figure 2). The highest rate of intact MII oocytes was observed in the culture of 10mM L-NAME.

DISCUSSION

In the study, the presence of all three NOS isoforms (eNOS, nNOS, iNOS) and their role in ageing porcine oocytes *in vitro* was demonstrated. The NOS isoforms occurred in matured and ageing oocytes. According to Hattori et al. (2000), mRNA NOS is present in matured mammal oocytes.

Although mRNA NOS and NOS are detected during oocyte growth and meiotic maturation in mice (Jablonka-Sharif and Olson, 1997; Abe et al., 1999), cattle (Tesfaye et al., 2006), and pigs (Chmelíková et al., 2010), NOS de novo proteosynthesis (according to the mRNA pattern) or the persistence of proteins synthesized in earlier oogenesis stages is uncertain in aged oocytes. However, significant NOS isoform relocalizations observed during oocyte ageing indicate the important role this enzyme plays in the ageing process.

The foci of eNOS were localized in the cytoplasm of aged oocytes. The findings are in agreement with eNOS localization in maturing porcine oocytes (Chmelíková et al., 2010). The fact that nNOS and iNOS are enclosed by the cytoplasmic membrane

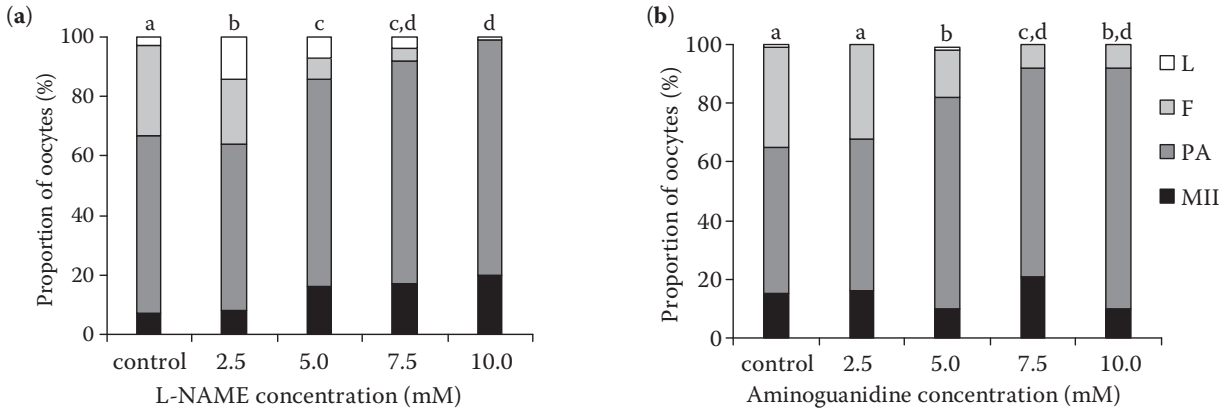

Figure 2. Influence of (**a**) L-NAME and (**b**) aminoguanidine on porcine oocytes aged for 72 h

MII = intact MII oocytes, PA = parthenogenetic activation, F = fragmentation, L = lysis
bars with different superscripts indicate significant differences between the ratios of aged oocyte stages ($P < 0.05$)

could be due to the known affinity of the actin cytoskeleton component for these two isoforms (Brophy et al., 2000; Zeng and Morrison, 2001). In spontaneously parthenogenetically activated oocytes, nNOS occurred mainly in the pronucleus. The nNOS localization in the cell nucleus is also known in somatic cells, and it is associated with transcription regulation (Yuan et al., 2004). For the genome reactivation of porcine embryos occurring in later embryonic development, nNOS localization may be either a form of parthenogenetic zygote preparation for embryonic genome reactivation or a reflection of early transcription of the same genes prior to complete genome reactivation (Latham and Schultz, 2001; Barnetová et al., 2012).

The signal intensity decrease of NOS isoforms in aged porcine oocytes was observed. After 24 h of prolonged cultivation, nNOS and iNOS levels decreased drastically. The decrease in eNOS level was less abrupt, and significant differences were determined after 72 h of prolonged cultivation. The reason for the various rates of isoform decrease is uncertain. Due to the fact that nNOS and iNOS levels in oocytes were relatively low and eNOS indicated a higher-intensity signal, the differences in the signal decrease rate may have resulted from diverse levels of specific isoforms (Kim et al., 2005; Chmelíková et al., 2010). Accordingly, detectable iNOS and nNOS signals in oocytes have yet to be observed (Kim et al., 2005; Hattori and Tabata, 2006). Moreover, the different localizations of NOS isoforms in oocytes and/or the effects of other substances with the ability to influence the half-life of NOS can play important roles in oocyte ageing (Ramet et al., 2003).

NOS isoforms play an essential part in meiotic maturation (Tao et al., 2005; Chmelíková et al., 2010). Our experiments highlight the role of NOS during prolonged cultivation of porcine oocytes. The ageing process is influenced by inhibitors, L-NAME, and the utilization of aminoguanidin. After 72 h of prolonged cultivation, most of aged oocytes remained either intact in MII phase or parthenogenetically activated. Presumably, the effect of NOS inhibition consisted in programmed cell death and lysis suppression. A similar impact was described for aminoguanidine in pancreatic β-cells of the islets of Langerhans (Corbett and McDaniel, 1996). Therefore, the higher percentage of parthenotes was not a result of NOS inhibition and NO absence, but rather of apoptosis or lysis inhibition. Conversely, recent studies have established the activation ability of NO donors (Petr et al., 2010).

Futher experiments are necessary to elucidate the physiological effects of NOS and to better understand the roles of NO in oocytes and somatic cells.

CONCLUSION

All three NOS isoforms are present in aged porcine oocytes, although their levels decrease during prolonged cultivation. NOS has the potential to play a role in physiological functions of porcine oocytes during *in vitro* ageing. Further experiments are necessary to definitely confirm this role.

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Corresponding Author

Ing. Jan Nevoral, 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
Tel. +420 224 382 932, fax +420 234 381 841, e-mail: nevoral@af.czu.cz