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Markery zrání savčích oocytů *in vitro* – vliv endokrinního disruptoru bisfenolu S

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doktorská disertační práce

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Prohlášení

Prohlašuji, že jsem disertační práci na téma: Markery zrání savčích oocytů *in vitro* – vliv endokrinního disruptoru bisfenolu S vypracovala samostatně a použila jen pramenů, které cituji a uvádím v přiloženém seznamu použité literatury.

V Praze dne: ……

podpis autora

Poděkování

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Obsah

1. Úvod

Meiotické zrání savčích oocytů je jedinečné asymetrické buněčné dělení, během kterého vzniká sérií po sobě jdoucích změn plnohodnotná samičí gameta, předurčená k oplození a zdárnému vývoji embrya. Mezi klíčové děje, nezbytné pro úspěšný průběh meiotického zrání, patří například rozpad jaderné membrány oocytu po výstupu z meiotického bloku, epigenetické modifikace nukleozómů nebo zarovnání chromozomů do metafázní roviny pomocí meiotického vřetene a následné vydělení pólového tělíska.

K negativnímu narušení funkcí reprodukční soustavy, zahrnující i proces meiotického zrání, může docházet vlivem toxických látek, které se vyskytují v životním prostředí a odtud vstupují do organizmu. Jednou z nejvýznamnějších skupin těchto exogenních chemikálií jsou endokrinní disruptory (EDs), které jsou schopné imitovat účinky některých endogenních hormonů a tím narušovat endokrinní systém, včetně hormonálně řízené reprodukční soustavy. Mezi látky ze skupiny EDs, významně zasahující do regulace fyziologických procesů reprodukce se řadí bisfenoly, které mají díky své nepostradatelné úloze při výrobě plastů a epoxidových pryskyřic široké uplatnění například v potravinářském průmyslu.

V současné době patří mezi nejvyužívanější bisfenoly bisfenol S (BPS), který má řadu vlastností, díky nimž byl zvolen jako vhodný komponent při výrobě obalových hmot využívaných v potravinářském průmyslu. Je stabilní při vystavení vysokým teplotám a odolný vůči slunečnímu záření. Tyto vlastnosti jsou přínosné pro stabilitu materiálů a BPS díky nim není tak náchylný k uvolňování z těchto hmot. Zároveň to ale znamená, že je chemicky stabilnější a hůře biologicky odbouratelný i po průniku do organismu člověka.

V současné době existuje jen velmi omezený počet publikovaných dat, zabývajících se negativními účinky BPS přítomného v organismu na reprodukci lidí. K dispozici je však vzrůstající počet studií prováděných v podmínkách *in vitro* a *in vivo*, zaměřujících se na mechanismus účinku BPS v organismu bezobratlých, ryb a savců, které potvrzují jeho endokrinně disrupční účinky a negativní vliv na reprodukci tak, jak je tomu u jiných bisfenolů.

V této práci jsme jako experimentální model zvolili myš (*Mus musculus*), která je z důvodu svého rychlého generačního cyklu vhodným modelem pro studii transgeneračních efektů a prase (*Sus scrofa*), často využívané jako biomedicínský model.

2. Literární rešerše

2.1. Oogeneze

Oogeneze je složitý děj, při kterém z původních primordiálních zárodečných buněk (primordial germ cells – PGCs) vznikají sérií změn zralé, oplozeníschopné pohlavní buňky – oocyty, které jsou nezbytné pro úspěšnou reprodukci savců, včetně člověka. Ústřední role oocytů v biologii je doložena i slavným výrokem *„Omne vivum ex ovo"* – veškerý život z vejce, který je připisován Williamu Harvey (*Exercitationes de Generatione Animalium*, 1651).

Proces, označovaný jako oogeneze, začíná u savců již v ranné fázi prenatálního vývoje a lze jej rozdělit do tří po sobě jdoucích fází – fázi množení, růstu a meiotického zrání (Wassarman and Albertini, 1994).

2.1.1. Množení oogonií

Vývoj pohlavních buněk samic savců začíná již v raném embryonálním vývoji a je ukončen až v dospělosti, v období zástavy pohlavní aktivity samice. Na počátku oogeneze, v proximálním epiblastu embrya, dochází k selekci skupiny buněk, která utvoří populaci potenciálních prekurzorů zárodečných buněk. Tato populace dále pasivně migruje k povrchu žloutkového váčku a začleňuje se mezi entodermální buňky v blízkosti allantois. Zde dochází k druhému cyklu molekulární selekce, po níž zůstává vyčleněna skupina buněk, které lze již identifikovat jako prekurzory zárodečných buněk, neboli PGCs. PGCs poté rychle proliferují a migrují přes žloutkový váček a zadní konec endodermu na kaudální konec embrya, následně již pomocí améboidních pohybů migrují přes dorzální mezenterium na budoucí místo primitivních gonád – genitální lišty (Wylie, 1993; Picton *et al*., 1998; Richardson and Lehmann, 2010). Kolonizace genitálních lišt probíhá těsně po tom, co tyto struktury vznikají z centrálního mezodermu a probíhá determinace somatického pohlaví (Boowles and Koopman, 2007). Aktivní pohyb určitým směrem, který PGCs vykazují v posledním úseku migrace, je dán jejich schopností reagovat na chemotaktické podněty. Tyto podněty vyvolávají látky (chemokiny), které jsou vylučovány přímo ze základu gonád a mimo navádění PGCs zároveň ovlivňují jejich mitotickou aktivitu. PGCs díky tomu mění svůj tvar a stávají se méně pohyblivými (Hurk and Zhao, 2005). Migrace PGCs je u prasete zahájena 18. den

embryonálního vývoje a vstup do genitální lišty je uskutečněn 26. den (Takagi *et al*., 1998). Oproti tomu u myšího embrya je migrace PGCs zahájena již 9. den vývoje a k dosažení genitální lišty dochází u většiny PGCs 11. den (Ewen and Koopman, 2010). Po usídlení v základu gonád ztrácejí PGCs schopnost pohybovat se úplně (Picton *et al*., 1998). Dochází k diferenciaci PGCs v oogonie, které jsou již pohlavně determinovány a začínají se intenzivně mitoticky dělit (Picton *et al*., 1998; Bowles and Koopman, 2007).

Časový interval, během kterého se oogonie mitoticky dělí, je druhově specifický. Nejvyššího počtu oogonií je u prasete dosaženo přibližně 50. den embryonálního vývoje, přičemž mitotickou aktivitu oogonie vykazují až do 7. dne po narození (Hunter, 2000; Kanitz *et al*., 2001). Oogonie myší jsou mitoticky aktivní již od 11. dne po oplození a nejvyšší aktivity dosahují den 13. K ukončení mitotického dělení dochází 14. den vývoje v souvislosti s pohlavní diferenciací gonád. Po ukončení mitotického dělení je počet oogonií konečný, po zbytek prenatálního a postnatálního života jedince už k dalšímu množení zárodečných buněk nedochází (Ewen and Koopman, 2010). Toto obecné dogma o konečném počtu oogonií je rozporováno tzv. teorií postnatální obnovy oocytů, která bude popsána níže.

Zahájení meiotického zrání je asynchronní a je uskutečněno ještě v průběhu prenatálního období (u prasat mezi 35. – 40. dnem embryonálního vývoje, u myší 13. – 16. den vývoje), kdy během posledního mitotického dělení dochází k finální replikaci DNA a k tvorbě vrstvy pregranulózních buněk, obklopující spolu s bazální membránou zárodečnou buňku. Následně zárodečné buňky, nyní již nazývány oocyty, vstupují do profáze prvního meiotického dělení (profáze I), která je dále členěna do 5 – ti po sobě jdoucích fází. Jsou jimi leptotene, zygotene, pachytene, diplotene a diakineze. V průběhu těchto fází dochází k postupné kondenzaci chromozomů, jejich homologním párování a tvorbě bivalentů, mezi nesesterskými chromatidami dochází ke crossing – overům, tedy ke vzniku genetických rekombinací. Místa, ve kterých došlo k překřížení chromatid, se nazývají chiasmata a jsou viditelná až do pozdní profáze (Hurk and Zhao, 2005; Ewen and Koopman, 2010; Cohen and Holloway, 2015). V poslední fázi profáze I dochází k oddálení rekombinovaných chromozomů a k zastavení meiózy v I. meiotickém bloku. Zárodečná buňka se od této chvíle označuje jako primární oocyt ve stádiu zárodečného váčku – GV (germinal vesicle – zárodečný váček) (Wassarman and Albertini, 1994; Hunt and Hassold, 2008). Jelikož je oogeneze prasat i myší

asynchronní, na vaječnících plodu se mohou vyskytovat v určité fázi vývoje jak mitoticky aktivní oogonie, tak meiotické oocyty (Bielanska – Osuchowska, 2006).

Teorie postnatální obnovy oocytů

Již v roce 1921 Pearl a Schoppe citovali "základní biologickou doktrínu“, že během postnatálního života savčích samic nemůže docházet k žádnému zvýšení počtu primárních oocytů nad rámec těch, které byly původně vytvořeny při vzniku vaječníku. Tato koncepce byla upevněna jako dogma v roce 1951 (Zuckerman) ve studii, která kriticky zhodnotila a účinně rozptýlila jakoukoli studii, která byla v rozporu s přesvědčením, že savčí samice mají během perinatálního období konečnou a neobnovitelnou zárodečnou rezervu. Předpokládalo se, že ačkoli samčí pohlaví si udržuje zárodečné kmenové buňky (GSC – germinal stem cells) pro spermatogenezi i během dospělého života, u samičího pohlaví v postnatálním vývoji neexistují kmenové buňky, které by se podílely na obnově pohlavních buněk. Počet oocytů pak klesá během postnatálního života prostřednictvím mechanismů zahrnujících apoptózu (Zuckerman, 1951).

Ačkoli toto dogma přetrvávalo více než 50 let, studie Johnson *et al*. (2004) později poskytla důkazy zpochybňující platnost této doktríny, která představuje jeden z nejzákladnějších pilířů reprodukční biologie. Johnson *et al*. (2005) ve své studii popisují jako potenciální zdroj zárodečných buněk pro obnovu oocytů kostní dřeň. Podle Bukovski *et al*. (2004) probíhá na vaječnících obnova zárodečných buněk díky přetrvávající přítomnosti primordiálních kmenových buněk, což může kompenzovat atrézii velkého množství folikulů a zajistit jejich relativně stálé množství v průběhu pohlavního života samice.

2.1.2. Růst oocytu

Pro získání schopnosti vystoupit z I. meiotického bloku a obnovit meiotické zrání, musí oocyt nejdříve podstoupit takzvanou „růstovou fázi“. Během růstu dochází ke kvalitativním i kvantitativním změnám buněčných kompartmentů oocytu a biochemickým změnám v cytoplazmě (Eppig *et al*., 1996; Hyttel *et al*., 1997). Typická je intenzivní transkripce, translace a syntéza a příjem makromolekul, které jsou v oocytu skladovány. Dále probíhá syntéza nových organel a modifikace organel stávajících. Tyto změny jsou nezbytné pro růst, vývoj a zrání oocytu a zároveň slouží jako zásoba informací a materiálu, potřebná

pro první období po oplození, před reaktivací embryonálního genomu (Schultz *et al*., 1979; Wassarman and Albertini, 1994).

Vlivem nárůstu syntézy mRNA a rRNA dochází v růstové fázi k přestavbě jádra oocytu, které se zvětšuje a jadérko nabývá kompaktnější podoby. Spolu s akumulací rRNA roste i množství ribozómů v polyzómech i množství polyzómů samotných, na kterých probíhá proteosyntéza z mRNA (Wassarman and Albertini, 1994; Picton *et al*., 1998; Hurk and Zhao, 2005). Pro regulaci uchovávání mRNA je důležitá polyadenylace (prodlužování poly – A konce mRNA) a deadenylace (zkrácení poly – A konce mRNA) (Reyes and Ross, 2016). Určité transkripty jsou syntetizovány pro okamžité použití, u jiných ale dochází k deadenylaci. To vede buď k jejich odbourání, nebo jsou určeny pro následné skladování. Skladování probíhá v ooplazmě v ribonukleových proteinech (ribonucleoproteins – RNP), nebo v RNA granulech. Transkripty skladované v RNP se nespojují s ribozomy, naopak dochází k asociaci s maskovacími faktory. Díky tomu nedochází k jejich okamžité translaci (Motlik and Fulka, 1986; Eichenlaub – Ritter and Peschke, 2002; Reyers and Ross, 2016).

Vlivem intenzivní proteosyntézy v oocytu se také zvětšuje objem endoplazmatického retikula a Golgiho komplexu (Oakberg, 1968; Wassarman and Albertini, 1994). Struktury Golgiho komplexu jsou z velké části přesunuty z oblasti kolem jádra na periferii k plazmatické membráně, kde se aktivně podílejí na exportu glykoproteinů do *zona pellucida* a formování kortikálních granul (Mehlmann *et al*., 1995; Hurk and Zhao, 2004). Též dochází k podstatnému zvýšení počtu mitochondrií a změně jejich struktury. Mění se tvar mitochondriálních krist a buněčná lokalizace mitochondrií, kdy dochází ke zvýšení množství mitochondrií v blízkosti plazmatické membrány (Wassarman and Albertini, 1994; Picton *et al*., 1998).

Jednou z nejdůležitějších změn, které v oocytu probíhají ve fázi růstu, je tvorba glykoproteinové vrstvy obklopující oocyt – *zona pellucida* (ZP), která představuje přirozenou bariéru mezi oocytem a kumulárními buňkami. Díky výběžkům kumulárních buněk, které prostupují skrz ZP, je zprostředkována komunikace mezi buňkami (Yanagimachi, 1988; Kanitz, 2001). V raných stádiích růstu oocytu glykoproteiny ZP vytvářejí ostrůvky jemných vláken nacházejících se mezi oocytem a kumulárními buňkami. Jak růst oocytu pokračuje, ZP se stává silnější a tvoří jí hustší síť propojených vláken, které plně obklopují oocyt a oddělují

ho od kumulárních buněk. Spojení pokračuje přes kontakty vzniklé mezi mikroklky oocytu a výběžky kumulárních buněk, které pronikají přes ZP. Na těchto kontaktech probíhá pomocí mezerových buněčných spojů (gap junctions) vzájemná výměna látek a komunikace prostřednictvím specifických buněčných signálů koordinující růst a zrání oocytu (Wassarman and Albertini, 1994; Soyal *et al*., 2000; Kanitz, 2001; Eppig, 2018).

Během fáze růstu získávají oocyty takzvanou meiotickou kompetenci. Tento proces umožňuje oocytu pozastavenému v I. meiotického bloku obnovit a dokončit proces meiotického zrání až do metafáze II. meiotického dělení (MII), kde nastává II. meiotický blok (Motlik *et al*., 1984).

Získání meiotické kompetence prasečího oocytu spočívá v úspěšném dokončení růstové fáze a dosažení plné velikosti, dále na protesyntéze a přítomnosti dostatečného množství regulačních faktorů meiotického zrání. Částečné meiotické kompetence nabývají oocyty již v průběhu růstu v době, kdy nejsou plně dorostlé. Tyto oocyty jsou schopné vystoupit z bloku a zahájit meiotické zrání, nejsou však schopné ho dokončit a zastavují zrání již v metafázi I. meiotického dělení (MI) (Yanagimachi, 1988). Částečně meioticky kompetentní prasečí oocyty jsou kromě své velikosti také charakteristické tím, že dekondenzovaný chromatin jádra je rozptýlen po celé nukleoplazmě a neobklopuje strukturu jadérka, které je ovšem dobře viditelné. Takovéto oocyty se nazývají non surrounded nucleolus (NSN; neohraničené jadérko). Po dosažení plné kompetence se chromatin kondenzuje a dochází k jeho soustředění kolem jadérka, kde tvoří souvislý prstenec. Takovéto oocyty se označují jako surrounded nucleolus (SN; ohraničené jadérko) (Fulka *et al*., 1986).

Rozměry, kterých oocyt v průběhu růstu dosahuje, odpovídají jednotlivým stupňům meiotické kompetence oocytu. Oocyty prasat jsou označovány jako meioticky nekompetentní, jestliže v průběhu růstové fáze dosahují velikostí 80 – 89 µm a 90 – 99 µm, částečné meiotické kompetence nabývají oocyty o velikosti 100 – 110 µm (NSN) a úplné meiotické kompetence nabývají oocyty o velikosti 120 – 125 µm (SN) (Motlik and Fulka, 1976).

Myší oocyty, oproti oocytům prasečím, jsou meioticky nekompetentní při velikosti menší, než 40 µm, částečné meiotické kompetence nabývají při velikosti 40 – 60 µm a plné meiotické kompetence dosahují ještě v průběhu růstu, při velikosti od 60 µm (NSN), kdy stále

probíhá intenzivní proteosyntéza. Plně dorostlé oocyty pak dosahují velikosti 83 – 90 µm (SN) (Wassarman and Josefowicz, 1978). Již oocyty ve stádiu neohraničeného jadérka tedy nabývají plné meiotické kompetence.

V době dosažení plné velikosti dochází u savčího oocytu k prudkému poklesu míry transkripce a oocyt se stává transkripčně neaktivním (Wassarman and Albertini, 1994; Picton *et al*., 1998).

2.2. Meiotické zrání

Po ukončení růstové fáze, těsně před ovulací, obnovují oocyty savců meiotické zrání, které zahrnuje přeměnu plně dorostlého meioticky kompetentního oocytu v oocyt zralý a oplozeníschopný. Tento složitý proces, jehož fyziologický průběh je nezbytný pro úspěšnou fertilizaci a následný embryonální vývoj jedince, je charakterizován dvěmi po sobě jdoucími děleními – I. a II. meiotickým dělením (Motlík and Fulka, 1986). V průběhu těchto dělení dochází paralelně k jadernému a cytoplazmatickému zrání, která se vzájemně ovlivňují. Jaderné zrání zahrnuje rozpad jaderné membrány, kondenzaci, přeskupení a redukci počtu chromozomů, spolu s vydělením pólového tělíska a dosažením metafáze II. Cytoplazmatické zrání umožňuje oocytu dokončit jaderné zrání a připravit ho na oplození a následný časný embryonální vývoj, na rozdíl od jaderného zrání probíhá i v době meiotického bloku. Je charakteristické dynamickými změnami a syntézou cytoplazmatických složek, jako jsou změny cytoskeletu a endoplazmatického retikula, změny v počtu, aktivitě a distribuci mitochondrií, množení glykogenových a lipidových složek oocytu, či syntéza kortikálních granul (Thibault *et al*., 1987; Hunter, 2000).

Před zahájením meiotického zrání je oocyt ve stádiu GV0 (NSN) s chromatinem ve formě dekondenzovaných vláken a jasně ohraničenou jadernou membránou. Výstupem z bloku je zahájeno I. meiotické dělení, pro které je charakteristický rozpad zárodečného váčku (GVBD – germinal vesicle break down) a kondenzace chromozomů, které vytvářejí homologní páry – bivalenty. Proces GVBD lze rozčlenit na 4 po sobě jdoucí fáze – GV1, GV2, GV3 a GV4. Ve fázi GV1 (SN) začíná chromatin kondenzovat, je viditelné jadérko, kolem kterého je soustředěn veškerý chromatin a vytváří kolem něj neporušenou prstencovou strukturu. Fáze GV2 je charakteristická narušením kompaktního prstence chromatinu

a vytvářením podkovovité struktury kolem jadérka. Ve fázi GV3 dochází k rozptýlení kondenzovaných chromatinových shluků z nukleolemy do celé nukleoplazmy, jadérko již není ohraničeno chromatinovým prstencem, je však stále viditelné. Ve fázi GV4 jsou přítomny shluky chromatinu v rozsahu celého jádra, jadérko však již zcela mizí a je dobře viditelná fragmentace jaderné membrány (Motlik and Fulka, 1976; Sun *et al*., 2016). Celý proces GVBD trvá u prasečích oocytů kultivovaných v *in vitro* podmínkách až 22 hodin, u myších oocytů 6 hodin (Motlik and Fulka, 1976; Fulka *et al*., 1986).

Po GVBD následuje metafáze I, během které se chromozomové bivalenty jako tetrády řadí v ekvatoriální rovině do metafázní destičky a dochází k přichycení bivalentů na tubulinová vlákna formujícího se dělicího vřeténka. Vazebným místem pro mikrotubuly jsou proteinové komplexy kinetochorů. Následuje anafáze I, chromozomální bivalenty se rozdělí díky deaktivaci synaptonemálního komplexu (proteinové struktury, která je přítomna uvnitř bivalentů a drží homologní chromozomy pohromadě) a homologní chromozómy se rozcházejí k opačným pólům buňky, tím je zabezpečena redukce počtu chromozomů a následně dochází k asymetrickému rozdělení cytoplazmy oocytu (Eichenlaub – Ritter, 2012; Holt *et al*., 2013).

Poslední fází I. meiotického dělení je telofáze I, kdy se vyděluje I. pólové tělísko, obsahující kromě chromozomů i malé množství organel (mitochondrie, ribozomy), snižuje se objem oocytu a vytváří se perivitelinní prostor. Po vydělení pólového tělíska nedochází k opětovnému sestavení jaderné membrány, ani replikaci DNA a oocyt rovnou vstupuje do II. meiotického dělení, kde ve stádiu metafáze II opět přerušuje zrání a vstupuje do druhého meiotického bloku. K dokončení II. meiotického dělení, tedy k podélnému rozštěpení zdvojených chromozomů a následnému vydělení II. pólového tělíska, dochází až po fertilizaci ovulovaného oocytu, nebo po partenogenetické aktivaci. Prasečí oocyty kultivované v podmínkách *in vitro* dosahují metafáze I po 24 hodinách a metafáze II po 44 – 48 hodinách kultivace (Yanagimachi, 1988; Wassarman and Albertini, 1994). Oocyty myší dosahují metafáze I po 8 hodinách a metafáze II po 16 hodinách kultivace v *in vitro* podmínkách (Nevoral *et al*., 2018; Cavalera *et al*., 2019).

2.2.1. Klíčové faktory meiotického zrání

Stejně jako mnoho dalších procesů v buňce, je i proces meiotického zrání regulován řadou faktorů, které mohou buď zabraňovat oocytu ve spontánním výstupu z meiotického bloku v nevhodnou dobu (inhibiční faktory) nebo se naopak svou aktivitou podílejí na znovuzahájení, průběhu a úspěšném dokončení meiotického zrání (Christmann *et al*., 1994; Liang *et al*., 2005).

Za udržení oocytu v I. bloku je odpovědná signální dráha cAMP/PKA (cAMP – cyklický adenosin monofosfát; PKA – cAMP dependentní proteinkináza A), přítomná v oocytech a kumulárních buňkách. Syntéza cAMP je katalyzována enzymem adenylát – cyklázou, lokalizovaným v cytoplazmatické membráně oocytu. Adenylát – cykláza svou aktivitou udržuje vysoké hladiny cAMP v oocytu a tím indukuje aktivaci PKA udržující oocyt v meiotickém bloku. Potlačením aktivity adenylát – cyklázy a aktivací fosfodiesteráz štěpících dvojnou vazbu cAMP, dochází ke snížení hladiny cAMP v oocytu (Dekel and Beers, 1980). Snížení hladin cAMP v oocytu může být také zapříčiněno přerušením mezibuněčných spojů gap junction mezi oocytem a kumulárními buňkami, čímž je omezen tok cAMP do oocytu z kumulárních buněk (Liang *et al*., 2007). Pokles koncentrace cAMP a aktivity PKA jsou nezbytné pro GVBD po znovuzahájení meiotického zrání (Dekel and Beers, 1980; Wassarman and Albertini, 1994).

Dalšími inhibičními faktory, které regulují setrvání oocytu v meiotickém bloku, jsou protein kináza C (PKC) a cyklický guanosinmonofosfát (cGMP). Aktivátory PKC inhibují změny v metabolismu fosfolipidů, které jsou spojeny se spontánní aktivací a znovuzahájením meiotického zrání. cGMP je tvořen kumulárními buňkami, kterými je pomocí gap junctions transportován do oocytu, kde svou činností zabraňuje degradaci cAMP (Norris *et al*., 2009).

K prolomení meiotického bloku a GVBD dochází v *in vivo* podmínkách důsledkem hormonální stimulace působením gonadotropinů – folikuly stimulujícího hormonu (FSH) a luteinizačního hormonu (LH). Receptory pro gonadotropiny nejsou v oocytech přítomny, proto je úspěch zrání závislý na distribuci LH signálu z okolních kumulárních buněk. Hormonální stimulace má poté za následek potlačení aktivity inhibičních faktorů meiotického zrání v kumulárních buňkách a oocytech (Eppig, 1991). V podmínkách *in vitro* je možné

u oocytů vyvolat spontánní zahájení zrání aspirací z folikulu, pro pokračování zrání pak následná kultivace za vhodných kultivačních podmínek, za využití FSH a LH v kombinaci se sérovými proteiny a růstovými faktory (Singh *et al*., 1997; Uhm *et al*., 1998). U prasete dochází k preovulačnímu zvýšení hladiny LH 36 – 40 hodin před ovulací (Hunter, 2000), 12 hodin před ovulací dochází k preovulačnímu zvýšení LH u myší (Liang *et al*., 2008).

Na znovuzahájení meiotického zrání a GVBD se také významně podílí proteinový komplex MPF (maturation promoting factor – faktor podporující zrání). Tento komplex se skládá z dvou podjednotek, z nichž jedna je regulační a druhá katalytická (Masui and Markert, 1971; Sorensen *et al*., 1985). Regulační podjednotkou MPF je cyklin B, katalytickou cyklin – dependentní proteinkináza 1 (Cdk1). Intracelulární hladiny cyklinu B a Cdk1 jsou zodpovědné za setrvání oocytu v profázi I, Cdk1 pak také za aktivitu MPF. V průběhu růstové fáze se hladiny těchto podjednotek v oocytu díky syntéze zvyšují, plně dorostlý meioticky kompetentní oocyt pak díky tomu obsahuje zásobu cyklinu B a Cdk1 v neaktivní formě, nazývající se pre – MPF (Gordo *et al*., 2001). Po aktivaci komplexu, MPF fosforyluje proteiny jaderné membrány, čímž indukuje její rozpad a pomocí pozitivní zpětné vazby aktivuje další MPF komplexy, čímž dochází k nárůstu kinázové aktivity (Nebreda *et al*., 1995).

Dalším nezbytným dějem, který vede po preovulační vlně LH ke znovuzahájení meiotického zrání, je aktivaceináz MAPK (mitogen – activated protein kinases – mitogenem aktivované proteinkinázy). MAPK patří do skupiny serin/threonin proteinových kináz a jsou aktivovány fosforylací aminokyselinových zbytků tyrosinu a threoninu. Jejich aktivita v prasečích oocytech narůstá krátce před rozpadem jaderné membrány (Alberts *et al*., 1998). MAPK se přímo podílejí na GVBD prostřednictvím aktivace MPF (Ohashi *et al*., 2003). Aktivní MAPK jsou důležité pro kondenzaci chromozómů a jejich segregaci během meiotického zrání (Kishimoto, 2003). Současně se podílejí na formaci dělícího vřeténka, jeho stabilizaci a elongaci, důležitých pro vydělení 1. pólového tělíska (Takenaka *et al*., 1998; Lee *et al*., 2000; Ohashi *et al*., 2003). Mimoto, MAPK potlačují opětovnou kompletaci jaderné membrány fosforylací jaderných laminů, tvořících proteinovou síť na vnitřní straně jaderné membrány (Inoue *et al*., 1998; Fan *et al*., 2002). V důsledku aktivních MAPK tak nenastává interfáze v přechodu mezi I. a II. meiotickým dělením, nedochází k replikaci DNA a k zahájení II. dělení ihned po I. dělení (Kishimoto, 2003; Fan and Sun, 2004). Aktivita MAPK přetrvává až

do dosažení metafáze II, kde se podílejí na udržování 2. meiotického bloku a vysoké aktivitě CSF (Maller *et al*., 2001; Kishimoto, 2003; Ohashi *et al*., 2003).

CSF je proteinový komplex, jehož součástí jsou také proteiny Emi1 a Mos (Li *et al*., 2002; Reimann and Jackson, 2002). Aktivita CSF vzrůstá po dokončení I. meiotického dělení a přetrvává do metafáze II (Takakura *et al*., 2005). Úloha CSF spočívá v potlačení aktivity APC/C, čímž zabraňuje polyubikvitinaci a proteolytické degradaci cyklinu B (Maller *et al*., 2001; Reimann and Jackson, 2002). Výsledkem je stabilizace MPF a udržování jeho konstantní aktivity (Maller *et al*., 2002; Fan and 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 MAPK (Kyriakis and Avruch, 2001).

Aktivita CSF je potlačena CaMKII, která je aktivována vzrůstem koncentrace iontů Ca2+ (Fan and Sun, 2004). Vzrůst koncentrace Ca2+ nastává po aktivaci oocytu spermií anebo po spontánní partenogenetické aktivaci (Lindemann and Goltz, 1986). K uvolnění Ca2+ dochází z intracelulárních depozit oocytu, jako jsou mitochondrie a endoplazmatické retikulum (Machaty *et al*., 1997).

2.2.2. Význam vybraných buněčných struktur v průběhu meiotického zrání

V průběhu meiotického zrání oocytů dochází k řadě klíčových dějů, které jsou nezbytné pro úspěšnou progresi zrání a následné oplození, vedoucí ke vzniku životaschopného embrya. Mezi tyto děje řadíme přeuspořádání a epigenetické modifikace jaderných nukleozómů, základních jednotek chromatinu, které jsou tvořeny DNA a histony a dále také rozpad jaderné membrány, na kterém se významně podílejí cytoskeletární složky oocytu, jako jsou proteiny vnitřní jaderné membrány – jaderné laminy (Link *et al*., 2018).

Epigenetické modifikace nukleozómů

Vývoj savčích oocytů je koordinován dynamickou regulací epigenetických modifikací nukleozómů, které propůjčují DNA další úroveň informací, ovlivňujících přístupnost nukleotidové sekvence k jaderným procesům jako je genová transkripce, což je zásadní jak pro následný průběh meiotického zrání oocytů, tak pro časný embryonální vývoj (Kageyama

et al., 2007). Epigenom – souhrn epigenetických modifikací napříč genomem – tak představuje základní součást mechanismu, kterým jsou informace o genomu organizovány, přizpůsobovány a interpretovány (Endo *et al*., 2005). Některé epigenetické modifikace, zejména methylace cytosinu, mohou být rozmnoženy do dceřiných buněk při replikaci DNA, aby se zajistila dlouhodobá paměť dřívějších rozhodnutí buňky, například genová aktivace nebo represivní události během specifikace linie, zatímco jiné jsou dynamičtěji kontrolovány v reakci na vnější signály. Epigenetické modifikace tedy diktují funkci genomu, ale také poskytují ukazatele vývojové historie, zkušeností nebo měnícího se vnějšího prostředí a díky tomu mohou nařídit nebo omezit pozdější reakce (Endo *et al*., 2005; Gu *et al*., 2010; Ge *et al*., 2015).

a.) Methylace DNA

Methylace DNA je nezbytnou součástí epigenetických modifikací během vývoje savčího genomu, jelikož hraje klíčovou roli v transkripční regulaci, jako je inaktivace chromozomu X, genomový imprinting a inaktivace transpozonů (Li, 2002; Santos and Dean, 2004; Morgan *et al*., 2005). Hlavním místem pro epigenetickou modifikaci DNA je methylace cytosinu v poloze 5 pyrimidinového kruhu CpG (cytosin trifosfát deoxynukleotid – fosfodiester – guanin trifosfát deoxynukleotid) dinukleotidů (Robertson and Wolffe, 2000), přičemž oblasti bohaté na CpG se nazývají CpG ostrovy. U samčích a samičích zárodečných linií zahrnují CpG ostrovy imprintované, specifické methylované regiony, které získávají methylaci v zárodečné linii a zachovávají tuto monoalelickou methylaci po celou dobu vývoje a postnatálního života (Sasaki and Matsui, 2008). Tyto lokusy odolávají celogenomové demethylaci, ke které dochází po oplodnění, a selhání získání nebo udržení methylace při imprintingu specifických methylovaných regionů vede k embryonální letalitě (Kaneda *et al*., 2004; Smith and Meissner, 2013). Mechanismy odpovědné za cílení methylace DNA na CpG ostrovy, zejména *in vivo*, zůstávají navzdory své důležitosti zatím málo objasněny. Je však známo, že methylace DNA pozitivně a negativně koreluje s řadou posttranslačních modifikací histonů, což naznačuje, že modifikace histonů mohou během procesů *de novo* nasměrovat komplex DNA methyltransferázy, a to i na určité CpG ostrovy (Cheng and Blumenthal, 2010; Blackledge and Klose, 2011).

V průběhu oogeneze myší dochází k methylaci DNA v nedělících se buňkách, na převážně nemethylovaném genomu, což z oogeneze činí vysoce informativní model pro zkoumání toho, jak mohou modifikace histonů formovat methylom DNA (Kobayashi *et al*., 2012). Například mohou histonové modifikace zpřístupnit nebo znepřístupnit chromatin pro komplex DNMT3A / DNMT3L [DNA (cytosin 5) methyl – transferáza 3α; DNA (cytosin 5) methyl – transferáza 3 – like]. Trimethylace a dimethylace H3K4 (K4me3 / K4me2) jsou charasteristické pro oblasti CpG ostrovů a označují místa iniciace transkripce (Deaton and Bird, 2011; Henikoff and Shilatifard, 2011), spolu s tím ale také eliminují činnost komplexů DNMT3A a DNMT3L prostřednictvím svých domén ATRX – DNMT3A – DNMT3L, čímž účinně chrání přidruženou DNA před methylací (Ooi *et al.,* 2007; Noh *et al*., 2015), histon H3K4 musí být tedy před započetím *de novo* methylace v přilehlém CpG ostrově demethylován. Naopak H3K27me2 může komplexy DNMT3A (Dhayalan *et al*., 2010) a DNMT3B [DNA (cytosin 5) methyl – transferáza 3β] aktivovat (Baubec *et al*., 2015; Morselli *et al*., 2015). Jelikož většina CpG ostrovů, které jsou v oocytu methylovány je intragenní, může akumulace K36me3 cílit na tyto lokusy pro methylaci DNA. Do nedávna byla většina analýz vztahu mezi modifikacemi histonů a methylací DNA prováděna v systémech, jako jsou buněčné embryonální kmenové buňky nebo spermie myší, vzhledem k relativní snadnosti izolace a velkému počtu takových buněk, které jsou k dispozici ve srovnání s oocyty (Smallwood *et al*., 2011; Kobayashi *et al*., 2012).

b.) <u>Histonový kód</u>

Jádro nukleozómu je složeno ze dvou kopií čtyř histonů (H2A, H2B, H3 a H4) tvořících oktamer, který je zabalen pomocí linkerového histonu H1 do struktur vyššího řádu. Histonové – COOH nebo – NH2 zbytky mohou být nahrazeny svými variantami nebo modifikovány prostřednictvím acetylace, ubikvitinace, methylace a fosforylace, za účelem regulace stavu chromatinu (Thompson *et al*., 2013; Watanabe *et al*., 2013; Hiragami – Hamada *et al*., 2016; Kadoch *et al*., 2016), který hraje zásadní roli v replikaci DNA (Franke *et al*., 2016), transkripci genů (Yang *et al*., 2016), homologní rekombinaci (Yoon *et al*., 2016), opravě dvouvlákenných zlomů (Lange *et al*., 2016) a stabilitě chromatinu. Tyto histonové varianty a posttranslační modifikace tvoří tzv. „histonové kódy“ (Wolfe *et al*., 2008; Tang *et al*., 2015). Široká škála histonových modifikací tvoří během meiotického zrání vysoce komplikovanou a dobře organizovanou regulační síť, což zajišťuje, že tento proces bezchybně

postupuje, a nakonec produkuje haploidní gamety. Dramatická přestavba chromatinových struktur během meiózy vytváří obrovské požadavky na posttranslační modifikace, varianty a přemístění histonů. Histonové kódy se tak nevyhnutelně řadí mezi ,,hlavní hráče'' v meiotickém zrání pohlavních buněk (Akiyama *et al*., 2004; De La Fuente *et al*., 2017).

Nejlépe studovanou epigenetickou modifikací histonů ve specifických zbytcích je methylace, která se obvykle vyskytuje na zbytcích lysinu a argininu histonů H3 a H4 a má zásadní roli jak při transkripční represi, tak při aktivaci během embryogeneze a embryonálního vývoje (Zhang and Reinberg, 2001; Kouzarides, 2002; Bedford and Clarke, 2009; Lan and Shi, 2009). Některé zbytky na histonu H3 a H4 mohou být mono –, di – nebo tri – methylované, což vede ke zvýšení komplexnosti epigenetické regulace. Tyto modifikace jsou katalyzovány histon – methyltransferázami (HMT) (Santos – Rosa *et al*., 2002; Wang *et al*., 2003), které využívají S – adenosylmethionin jako svého dárce methylové skupiny k úpravě zbytků argininu nebo lysinu, a tím regulují transkripci genů, integritu genomu a epigenetickou dědičnost (Bannister *et al.,* 2002; Klose and Zhang, 2007; Wasserzug – Pash and Klutstein, 2019). Tyto modifikace jsou považovány za markery buď pro aktivaci nebo represi příbuzných genů (Martin and Zhang, 2005), mezi nimiž jsou methylace H3K4, H3K36 a H3K79 spojeny s aktivací genů, zatímco methylace H3K9, H3K27 a H4K20 jsou odpovědné za represi genů. Ve skutečnosti hraje methylace histonů životně důležitou roli v pachytenním stádiu meiózy tím, že moduluje expresi genů souvisejících s meiotickým zráním (Martin and Zhang, 2005; Klose and Zhang, 2007; De La Fuente *et al*., 2017).

Jaderná lamina

Jaderná lamina je pevná, vláknitá proteinová síť na jaderné periferii, která je formována cytoskeletárními proteiny patřícími do skupiny intermediálních filament, nazývající se laminy. Tato síť obklopující nukleoplasmatický povrch vnitřní jaderné membrány definuje mechanické vlastnosti jádra a též je zapojena do replikace DNA, transkripce a organizace chromatinu během meiotického zrání (Aebi *et al*., 1986; Dechat *et al*., 2009; Link *et al*., 2018).

V savších buňkách, včetně buněk pohlavních, dochází k expresi dvou typů laminových proteinů (LMN) o různých izoformách, které se samostatně organizují do struktur vyššího řádu a utváří tak jadernou laminu (Prather *et al*., 1989). V nedávných studiích na myších

oocytech bylo prokázáno, že malé, avšak významné frakce LMN se během fáze GV (GV0, NSN) a časné fáze GVBD (GV1 – GV2, SN) vyskytují kromě oblasti jaderné laminy také v blízkosti jadérka a bývají označovány jako nukleoplazmatické laminy (Susor *et al*., 2015; Pochukalina *et al*., 2016).

Na základě biochemických a sekvenčních charakteristik lze LMN v oocytech savců členit na laminy typu B (LMNB), jejichž izoformy jsou lamin B1, lamin B2 a lamin B3 (krátký protein specifický pro zárodečné buňky), kódované geny *LMNB1* a *LMNB2* a dále na laminy typu A (LMNA), jejichž izoformy jsou lamin A, lamin C, lamin AA10 a lamin C2 (specifický pro zárodečné buňky) a jsou kódovány genem *LMNA*. Jak bylo popsáno v somatických buňkách a později i v oocytech *Xenopus Leavis* (Goldberg *et al*., 2008) a myší (Pochukalina *et al*., 2016), oba typy LMN tvoří v jaderné lamině oddělené vláknité sítě s velmi omezeným počtem kolokalizačních bodů a vykazují organizaci domén typu intermediálních filament s N – terminální hlavovou doménou, centrální α – helikální tyčovou doménou a globulární C – terminální koncovou doménou. Zatímco koncové domény LMNB jsou permanentně fernesylovány a karboxymethylovány a proto zůstávají pevně spojeny s jadernou membránou, koncové domény LMNA podléhají dalšímu štěpení, což je činí více rozpustnými a mobilními, na rozdíl od LMNB, které jsou imobilní (Shimi *et al*., 2008; Goldberg *et al*., 2008; Koncicka *et al*., 2020).

a.) Dynamika LMN v průběhu meiotického zrání

Oocyty v časném stádiu GV (GV0) jsou charakteristické difuzním rozptýlením euchromatinu, heterochromatinem soustředěným do chromocenter a nepřítomností chromatinového prstence. V tomto stadiu jsou LMNA lokalizovány pouze na obvodu jádra, kde na vnitřní jaderné membráně tvoří jadernou laminu (Schatten *et al*., 1985; Prather *et al*., 1989). LMNB jsou kromě jaderné laminy přítomny i v nukleoplazmě, v oblasti chromocenter, na jejichž formování se podílejí (Pochukalina *et al*., 2016). Výstup oocytu z prvního meiotického bloku a znovuzahájení meiotického zrání je spojeno se změnami v intracelulární distribuci jaderných LMN. Ve stádiu GV1 (SN), kdy se v oocytu formuje chromatinový prstenec po obvodu jadérka, tvořený kondenzovaným chromatinem, aktivní komplex MPF začíná postupně fosforylovat LMN. Vlivem toho LMNA depolymerizují a částečně disociují z jaderné membrány a poté jsou lokalizovatelné nejen na vnitřní jaderné membráně, jako

součást jaderné laminy, ale také na periferii prstence. Tvoří tak v oblasti jádra a jadérka dvě koncentrické kružnice, odpovídající jadernému obalu a chromatinovému prstenci. Membránové LMNB jsou díky své struktuře imobilní, zůstávají tedy připojeny k jaderné membráně až do pozdní fáze GVBD (GV4). U myších experimentálních modelů, které byly dosud využity pro studium dynamiky LMN v průběhu zrání savčích oocytů, nebyly lokalizovány žádné typy LMN ve vnitřním prostoru jadérka (Susor *et al*., 2015; Nikolova *et al*., 2017).

Postupná disociace LMNA z jaderné laminy díky fosforylaci MPF vede také k oddělení kondenzovaného chromatinu z vnitřní jaderné membrány. Ve stádiu GVBD pak mohou LMNA vytvářet agregáty nepravidelného tvaru v blízkosti chromozomů (Prentice – Biensch *et al*., 2012). Až ve fázi GV4 dochází k depolymeraci LMNB, které zůstávají připojeny k membráně, ale již ji nemohou poskytovat podporu, což vede k rozpadu jaderného obalu na malé vezikuly (Dechat *et al*., 2008). Sanfins *et al*. (2004), detekovali LMNB u myších oocytů a popsali počáteční kolaps jaderné laminy, následovaný postupným roztažením reaktivních struktur LMNB v prometafázi.

Během metafáze I a metafáze II nedochází k asociaci LMN s kondenzovanými chromozomy, avšak nedávné studie prokázaly, že se jak LMNA, tak LMNB, asociují s meiotickým vřetenem. Tato asociace je slabší u LMNA, které jsou detekovány v celé cytoplazmě, avšak s tendencí soustředit se do difúzního kruhu v oblasti obklopující vřeteno, bez vazby na samotná vlákna. Oproti tomu LMNB vykazují společnou lokalizaci s meiotickým vřetenem. Kolokalizace je plná a prodloužená po celé délce vláken vřetene (Susor *et al*., 2015; Nikolova *et al*., 2017).

Disociované LMN se opět formují a polymerizují na vnitřní jaderné membráně až v průběhu oplození, v pronukleární zygotě, kde byla pozorována jaderná lamina s typickou strukturou a složením obsahujícím oba typy LMN (Prather *et al*., 1989; Foster *et al*., 2007; Kelly *et al*., 2010). Při sestavování jaderného obalu a jaderné laminy LMN zpočátku dimerizují prostřednictvím svých tyčových domén a následně polymerizují ve směru od hlavové, ke koncové doméně (“head – to – tail orientation“). Tím se utváří antiparalelní sestavení polymerů LMN, z nichž se formují protofilamenta, která se dále spojují za vzniku intermediálních filament (Heitlinger *et al*., 1991; Karabinos *et al*., 2003; Foeger *et al*., 2006).

K expresi nového LMNB v průběhu embryogeneze však dochází podstatně dříve, než je tomu u LMNA. Zatímco LMNB je exprimován již po zahájení transkripce v embryonálním genomu, LMNA je exprimován až po zahájení embryonální tkáňové diferenciace (Adam *et al*., 2012).

b.) Interakce lamin – chromatin

Jaderná lamina je vysoce organizovaná trojrozměrná síť, která vytváří přímá i nepřímá spojení s chromatinem na mnoha různých úrovních. Významně se tak podílí na pohybu chromozomů v průběhu profáze meiózy I, který je nepostradatelný pro jejich zarovnání, homologní párování a následnou segregaci. Bylo prokázáno, že postupná fosforylace a depolymerace LMN jaderné laminy po zahájení meiotického zrání koresponduje s oddělováním chromatinu od jaderné membrány, čímž je podpořena reorganizace chromatinu v průběhu meiotického zrání (Guelen *et al*., 2008; Link *et al*., 2018).

Redistribuce LMNA z jaderné membrány a kolokalizace nukleoplazmatického LMNB s chromocentry, poukazuje na interakce těchto proteinů s kondenzovaným chromatinem nejen v oblasti jaderného obalu, kde jsou před vystoupením oocytu z meiotického bloku dlouhodobě známé interakce lamin – chromatin, ale také uvnitř jádra, v oblasti chromatinového prstence a kondenzovaných shluků heterochromatinu (Dechat *et al*., 2008; Pochukalina *et al*., 2016).

Specifické genomové oblasti, které jsou přednostně lokalizovány na jaderné periferii v oblasti vnitřní jaderné membrány, kde vytvářejí přímý molekulární kontakt s LMN, se nazývají chromozomové domény asociované s jadernou laminou (LAD; lamina – associated domains). Velké množství genů v LAD je během interfáze exprimováno ve velice nízkých hladinách, což naznačuje úlohu LMN v genové represi oocytů, která byla již prokázána u somatických buněk. Mezi nepřímá spojení LMN a chromatinu se řadí spojení s jadernými póry vázanými na chromatin, nebo spojení LMN s LEM doménami proteinů (LEM; Lap2 – emerin – MAN1), které jsou přes komplex BAF (barrier to autointegration factor) propojeny s chromatinem. LEM domény jsou strukturní části tří proteinů – LAP2 (lamin – associated polypeptide; polypeptid asociovaný s LMN), emerinu a MAN1, které jsou integrované do jaderné laminy, zprostředkovávají její kontakt s chromatinem a tím se zapojují do reorganizace chromatinu a rozpadu jaderné membrány v průběhu zrání (Yáñez – Cuna and van Steensel, 2017; Cohen – Fix and Askjaer, 2017; Link *et al*., 2018).

Chromozomové pohyby v jádře, na nichž se LMN podílejí, jsou řízeny cytoskeletárními silami generovanými v cytoplazmě a přenášenými jadernou membránou do nukleoplazmy na telomery chromozomů prostřednictvím konzervovaného mechanismu zahrnujícího LINC komplexy (linker of nucleoskeleton and cytoskeleton complex, spojovací komplex nukleoskeletu a cytoskeletu). Tyto komplexy jsou tvořeny doménovými proteiny SUN a KASH, které jsou přítomné na vnitřní (SUN) a vnější (KASH) jaderné membráně a fyzicky interagují v lumenu perinukleárního prostoru jaderné membrány. SUN domény jsou zároveň přímo, nebo nepřímo propojeny s LMN (Zeng *et al*., 2017).

Pohyb chromozomů zprostředkovaný LINC komplexy se shoduje s drastickou reorganizací jaderné membrány a koreluje s fosforylací a disociací LMN z jaderné membrány v průběhu meiotického zrání. Poruchy v disociaci LMN vedou k zpožděnému rozpadu jaderné membrány, ke změně v uspořádání chromatinu, nepárovým nebo vzájemně propojeným chromozomům a ke zpomalení pohybu chromozomů. Kinázy fosforylující LMN jsou přenášeny na LMN přes telomery chromozomů připojené k jaderné membráně, disociace a rozvolnění sítě LMN tedy hraje roli při modulaci kontaktů mezi chromozomy a jadernou membránou během meiózy (Link *et al*., 2018).

2.3. Exogenní látky negativně ovlivňující organismus savců

V životním prostředí se přirozeně, nebo vlivem lidské činnosti vyskytuje široké spektrum látek, které mohou být přírodního, nebo syntetického charakteru a díky svým vlastnostem se v lidské společnosti začaly využívat k mnoha různým účelům, aniž by se blíže zkoumal jejich mechanismus účinku, vliv na životní prostředí, nebo organismus člověka, včetně reprodukčních funkcí. Bohužel u mnoha z těchto látek byl později zjištěn negativní účinek jak na životní prostředí, tak na organismus savců, člověka nevyjímaje. Mezi známé případy patří masové použití DDT jako insekticidu, thalidomidu jako léku pro těhotné ženy (McBride, 1961), novějších neonikotinoidních insekticidů používaných v ochraně rostlin (Blacquiere *et al*., 2012), nebo světově nejrozšířenějšího herbicidu – Roundupu, na bázi glyfosátu (Spinaci *et al*., 2020).

Postupně narůstající evidence o negativních účincích takových látek vedou k jejich následnému omezení a nahrazení jinými alternativami. V řadě případů to přináší skutečné

zlepšení (Landrigan *et al*., 2004), na druhé straně však existuje řada případů, ve kterých byly široce využity látky, jejichž negativní účinky na životní prostředí nebo lidské zdraví byly zjištěny až po dlouhém období užívání. Řada z nich přísluší do skupiny takzvaných endokrinních disruptorů (EDs). Výsledkem endokrinně disrupčního efektu této skupiny látek může být vedle celé řady dalších efektů i narušená hormonální bilance, čímž je velmi často ovlivněna i reprodukční soustava a její fyziologické fungování. Hodnocení reprodukčních funkcí a detailní studium vývoje pohlavních buněk tak může být jedním z vhodných ukazatelů, podle kterých lze odhadovat rizika endokrinních disruptorů na organismus živočichů, včetně člověka (Damstra *et al*., 2002).

2.3.1. Endokrinní disruptory

Pojem endokrinní disruptor (ED) – chemikálie narušující endokrinní systém, poprvé použila v roce 1991 Theo Colborn (World Wide Nature Fund Wingspread Conference, Wisconsin, USA, 1991) poté, co její tým identifikoval v lidském prostředí různé skupiny látek, které mají díky svým vlastnostem potenciál vstupovat do organismu živočichů a lidí a tím ovlivňovat hormonálně řízené soustavy, včetně soustavy reprodukční (Colborn *et al*., 1992). Oficiální stanovisko Endokrinologické společnosti USA (Endocrine Society of the US) k látkám s endokrinně disrupčním efektem bylo však vydáno až v roce 2009, kdy byly EDs charakterizovány jako "exogenní činitelé (látky), kteří zasahují do syntézy, sekrece, transportu, metabolismu, vazby nebo eliminace přirozených krevních hormonů, které jsou přítomné v těle a jsou zodpovědné za udržování homeostáze, reprodukce, vývinu a/nebo chování organismu" (Diamanti – Kandarakis *et al*., 2009).

Typickou vlastností EDs je tedy jejich hormonální aktivita, díky které mohou imitovat činnost endogenních hormonů. Tato aktivita a její výsledný vliv na organismus závisí na několika faktorech, jako je koncentrace EDs i endogenních hormonů v organizmu, délka expozice, nebo období, ve kterém je organismus účinkům EDs vystaven.

Účinky EDs na organismus mohou být potlačeny nebo mohou úplně vymizet v případě, že je jejich koncentrace v organismu vyšší, než fyziologická úroveň hormonu, jehož účinek mimikují. Tato schopnost EDs dosáhnout paradoxně silnějších účinků v nízkých, subtoxických dávkách než ve vysokých dávkách (vom Saal and Welshons, 2005) se nazývá

"efekt nízkých dávek" (low dose effect) (Grasselli *et al*., 2010; Vandenberg *et al*., 2012). Hypotéza efektu nízkých dávek předpokládá, že exogenní chemické látky, které vykazují hormonální aktivitu, mohou působit zcela specifickým způsobem a nelze u nich tedy uplatnit, *a priori* předpokládat tradiční toxikologické dogma, které tvrdí, že úměrně se vzrůstající dávkou chemické látky vzrůstá i toxický účinek (Vandenberg *et al*., 2012). Navíc, odezva organismu na expozici EDs se může se stoupající dávkou nelineárně měnit, což může například znamenat, že nejvyššího účinku EDs na organismus je dosaženo u nejnižší a nejvyšší použité dávky, zatímco dávky v rozmezí těchto dvou hodnot vykazují nejnižší efekt a *vice versa*. Odezva organismu na expozici stejnou dávkou EDs se může v závislosti na době expozice také nelineárně měnit – odpověď organismu je tedy nemonotónní. Proto byla definice nízké dávky rozšířena o takzvané "nemonotónní odezvy". Díky těmto poznatkům by tedy nemělo docházet k odmítání vědeckých studií zaměřujících se na účinky EDs jen proto, že výsledné závěry neodpovídají klasickému toxikologickému pojetí monotonie. Mechanismy odpovědné za nelineární účinky jsou detailně popsány (Vandenberg *et al*., 2012), obvykle v souvislosti s interakcí mezi ligandem (hormonem nebo ED) a hormonálním receptorem (Vandenberg, 2014). Nelineární odpovědi na dávku se běžně vyskytují u endogenních a syntetických látek (např. léků, hormonů, peptidů), které aktivují nebo inhibují signální dráhy zprostředkované receptory ovlivňující různé biologické funkce. Mohou tedy působit jako agonisté, ale i antagonisté endogenních hormonů, jelikož vazbou na hormonální receptory umocňují, nebo oslabují účinky endogenních hormonů v organismu (Calabrese and Baldwin, 2001; Calabrese, 2005).

Efekty nízké dávky jsou popsány u většiny EDs (Birnbaum, 2012; Zoeller *et al*., 2012; Vandenberg *et al*., 2012; 2013; Bergman *et al*., 2013; Demeneix *et al*., 2020) a mnoho z nich lze přičíst též období expozice. Expozice EDs během kritických období vývoje organismu může nevratně ovlivnit diferenciaci a organogenezi. Studie zabývající se expozicí EDs v průběhu vývoje také prokázaly, že EDs mění epigenom, což naznačuje, že změněná epigenetika může být jedním z mechanismů působení některých EDs (Vandenberg *et al*., 2013; Simeoni *et al*., 2018; Rattan *et al*., 2019).

Spojení EDs s negativními biologickými účinky u zvířecích druhů vyvolalo obavy, že nízkoúrovňová "subtoxická" expozice může způsobit podobné účinky i u lidí (Giesy *et al*.,

1994; Guillette *et al*., 1994; 1995; Fry, 1995; Sumpter and Jobling, 1995; Solla *et al*., 1998; Siegel *et al*., 2019). Negativní vliv některých EDs na lidský organismus byl později opravdu prokázán na mnoha úrovních. Například EDs významně ovlivňují reprodukci, včetně vývoje spermií (Li *et al*., 2011; Knez *et al*., 2014; Sharma *et al*., 2019) a oocytů, stejně jako následný embryonální vývoj (Mok – Lin *et al*., 2010; Xiao *et al*., 2011; Cabry *et al*., 2020). Kromě toho byl popsán účinek EDs na reprodukci dospělých osob včetně transgeneračního efektu (Susiarjo *et al*., 2015; Ziv – Gal *et al*., 2015; Rattan and Flaws, 2019). V současnosti široce rozšířené EDs, zasahující do regulace fyziologických reprodukčních procesů, představují bisfenoly.

2.3.1.1. Bisfenoly

Chemická struktura této významné třídy EDs je charakterictická přítomností dvou fenolických kruhů spojených dohromady uhlíkovým atomem. Tyto difenylalkany obdržely obecný název bisfenoly. Zástupců bisfenolů je několik, mezi nejvýznamnější patří bisfenol F (BPF), v jehož struktuře spojovací uhlíkový atom nemá žádný substituent, bisfenol A (BPA), kde spojovací uhlíková skupina obsahuje dvě methylové skupiny, bisfenol S, kde uhlíková skupina obsahuje navázanou sulfonylovou skupinu, nebo bisfenol AF, pokud jsou methylové skupiny bisfenolu A perfluorované. Bisfenoly patří v současné době mezi nejvyužívanější chemikálie v plastických hmotách, epoxidových pryskyřicích a/nebo polykarbonátech v potravinářském průmyslu. Další velice rozšířené využití našly bisfenoly u recyklovaného papíru a papíru pro termální tiskárny (Rosenmai *et al*., 2014; Wang *et al*., 2015).

Donedávna nejvíce využívaným bisfenolem v těchto materiálech byl BPA, jehož produkce činila v roce v roce 2016 8 milionů tun a předpokládá se, že v roce 2022 bude jeho produkce činit až 10,6 milionů tun (Abraham and Chakraborty, 2020). Bohužel však, jak bylo zjištěno až po dlouhé době jeho využívání, teplo, UV záření, alkalická úprava, intenzivní promývání, nebo kontakt s kyselými nebo bazickými sloučeninami urychlují hydrolýzu esterové vazby spojující molekuly BPA v polykarbonátu a pryskyřicích a to vede k uvolnění monomeru BPA, který tak vstupuje do životního prostředí, potravního řetězce a lidského těla, kde působí jako endokrinní disruptor (Rubin, 2011) s nepříznivým účinkem na lidské zdraví, včetně obezity, diabetu, abnormálního chování a ženských a mužských reprodukčních funkcí. Hlavní cestou expozice člověka je konzumace kontaminovaných potravin, pitné vody

nebo dermální kontakt s termálním papírem a kosmetikou, nebo inhalace kontaminovaného prachu ze vzduchu (Miyamoto and Kotake, 2005; Huang *et al*., 2012; Ribeiro *et al*., 2017). Není tedy překvapivé, že řada studií později prokázala přítomnost BPA v lidských tělních tekutinách, tj. moči, krevním séru, folikulární tekutině, plodové vodě, mateřském mléce a seminální plazmě (Yamada *et al*., 2002; Ikezuki *et al*., 2002; Liao *et al*., 2012; Mendonca *et al*., 2014; Vitku *et al*., 2015), ale také v životním prostředí. V dnešní době je již přítomnost BPA prokázána ve vzduchu, bytovém prachu, odpadních a podzemních vodách, nebo v řekách a v mořské vodě (Liu *et al*., 2009; Huang *et al*., 2012; Yuan et al., 2014; Desforges *et al*., 2014; Sun *et al*., 2016; Graziani *et al*., 2019; Caban and Stepnowski, 2020a; 2020b).

Vzhledem ke vzrůstajícímu počtu studií, které prokazují negativní vliv BPA na organismus člověka na mnoha úrovních, včetně reprodukce, je použití BPA v některých zemích omezeno nebo zakázáno (kojenecké lahve) (EFSA, 2011; FDA, 2012). K jeho nahrazení ve výrobním procesu se nyní využívají další bisfenoly s podobnou strukturou, a to ačkoliv chybí důkazy o účincích těchto bisfenolů na organismus člověka a jeho reprodukční soustavu. Jedním z nejčastějších substituentů je i BPS (Eladak *et al*., 2015).

2.3.1.2. Bisfenol S (4, 4' – sulfonyldifenol)

BPS má řadu vlastností, díky kterým byl zvolen jako vhodný komponent při výrobě obalových hmot využívaných v potravinářském průmyslu, ve kterém dochází k nejčastějšímu kontaktu s organismem člověka. Je stabilní při vystavení vysokým teplotám a odolný vůči slunečnímu záření. Tyto vlastnosti jsou přínosné pro stabilitu materiálů a BPS se díky nim z těchto hmot neuvolňuje do prostředí v takové míře, jako tomu bylo u BPA. Zároveň to ale znamená, že je chemicky stabilní a hůře biologicky odbouratelný i po průniku do organismu (Chen *et al*., 2002; Liao *et al*., 2012). Jako chemikálie, jejíž používání podléhá slabé legislativní regulaci, je nyní BPS používán v mnoha materiálech a jeho přítomnost byla prokázána v lidském organismu v mnoha populacích ekonomicky rozvinutých zemí (Liao *et al*., 2012).

V Evropské unii je roční produkce BPS až 10 000 t a neustále se zvyšuje (Del Moral *et al*., 2016). BPS v koncentracích podobných nebo dokonce větších, než BPA byl již zjištěn v abiotickém prostředí, jako jsou kaly z čistíren odpadních vod (Lee *et al*., 2015; Yu *et al*., 2015), povrchové vody (Yamazaki *et al*., 2015; Jin and Zhu, 2016), nebo bytový prach (Wang *et al*., 2015; Liu *et al*., 2021). V důsledku širokého výskytu byl BPS detekován v potravinách

(Liao and Kannan, 2014) a následně i v lidských tělních tekutinách. Nejvyšší koncentrace BPS v tělních tekutinách byly naměřeny u lidí, kteří jsou expozici BPS vystavováni pravidelně ve vyšší míře (pokladní), nebo u asijského obyvatestva, kde je koncentrace BPS v prostředí výrazně vyšší, než v jiných světových oblastech. Průměrné hodnoty BPS v moči se podle dostupných studií pohybují v rozmezí 0.67 ng / ml až 2.53 ng / ml (Chen *et al*. 2016; Ndaw *et al*., 2018; Philips *et al*., 2018), s tím korelují koncentrace ve folikulární tekutině – až 2.11 ng / ml (Dimitriadis *et al*., 2017), v krevní plazmě se koncentrace BPS pohybuje mezi 0.073 ng / ml a 4.844 ng / ml (Thayer *et al*., 2016; Macczak *et al*., 2017; Mokra *et al*., 2017), v seminální plazmě je to 0.12 ng / ml až 0.17 ng / ml (Smarr *et al*., 2018), v mateřském mléce až 0.683 ng / ml a v pupečníkové krvi až 0.12 ng / ml (Niu *et al*., 2017; Liu *et al*., 2017).

K expozici člověka dochází různými cestami, a to jak dermálním kontaktem, inhalací kontaminovaného vzduch a prachu, tak příjmem *per os*, jež tvoří největší podíl přijatého BPS (Wang *et al*., 2015; Thayer *et al*., 2016; Ndaw *et al*., 2018; Wan *et al*., 2018; Wu *et al*., 2018). V současné době existují jen velmi omezená data zabývající se negativními účinky BPS přítomného v organismu na reprodukci lidí, je však k dispozici vzrůstající počet studií prováděných v podmínkách *in vitro* a *in vivo*, zaměřujících se na účinky BPS a jejich mechanismus v organismu bezobratlých, ryb a savců.

Studie zabývající se mechanismy účinku BPS podle cílových struktur v buňkách zvolených modelových organismů (myš, potkan) a lidských buněčných liniích, prokázaly negenomické účinky BPS ovlivňující buněčnou signalizaci (Viñas and Watson, 2013a; 2013b). BPS se váže na sérové albuminy (Mathew *et al*., 2014) a proto je snadno distribuovatelný po celém těle. Také pro svou schopnost distribuce je BPS schopen napodobit vlastnosti hormonů a interagovat s ER (estrogenové receptory) (Delfosse *et al*., 2012; Rosenmai *et al*., 2014; Le Fol *et al*., 2015) a byla potvrzena jeho přímá vazba na jaderné ER (Yamasaki *et al*., 2004), podobně jako u dalších bisfenolů, čímž je schopný regulovat genovou expresi genomické cesty (Mesnage *et al*., 2017; Li *et al*., 2018). Ve femtomolárních až pikomolárních koncentracích indukuje BPS membránové ERα – zprostředkované dráhy a účinky na MAPK signalizaci, buněčnou proliferaci a aktivaci kaspázy 8 (Viñas and Watson, 2013a; 2013b). Tyto rychlé, negenomické dráhy jsou významné pro optimální funkci buněk, zprostředkování proliferace a apoptózy (Viñas and Watson, 2013a; 2013b), stejně jako pro další akce, jako

jsou funkce pankreatických buněk (Alonso – Magdalena *et al*., 2008) a estrogenem zprostředkované hypofyzární mozkové funkce a chování (Moenter and Chu, 2012; Laredo *et al*., 2014). BPS tedy vykazuje estrogenní aktivitu jako estradiol a je schopen stimulovat dráhy membránového receptoru, které jsou obvykle regulovány estradiolem a zasahuje do regulace steroidních genových transkriptů (Eladak *et al*., 2015; Feng *et al*., 2016). Tyto výsledky dokazují, že ER signalizace hraje klíčovou roli v reprodukční neuroendokrinní odpovědi na expozici BPS. Také byl prokázán obdobný mechanismus účinku BPS ve vztahu k androgenům (Kitamura *et al*., 2005; Molina – Molina *et al*., 2013; Rosenmai *et al*., 2014). Zhang et al. (2018) posléze prokázal vazbu BPS na thyroidní receptory, u kterých došlo k proliferaci sledovaných buněk GH3 u potkanů (Zhang *et al*., 2018).

Další významnou buněčnou drahou zasahující do reprodukce je dráha cytochromové aromatázy, která je ústředním prvkem syntézy estrogenů, katalyzující konečný krok omezující rychlost přenosu androgenů na estrogeny. Expozice nízké dávce BPS vyvolává předčasnou hypotalamickou neurogenezi, zprostředkovanou signalizací aromatázy, která je aktivována v odezvě na expozici látek s estrogenní aktivitou (Kallivretaki *et al*., 2006; Kinch *et al*., 2015). Navíc Qiu *et al*. (2016) popsali, že flavinový inhibitor aromatázy (FAD) významně zeslabuje stimulační účinky BPS na reprodukční neuroendokrinní expresi genů. Tento výsledek dokazuje, že enzymatická aktivita aromatázy je také částečně vyžadována pro zprostředkování stimulačních účinků BPS na reprodukční neuroendokrinní systém (Qiu *et al*., 2016).

U outbredních potkanů kmene Wistar bylo prokázáno, že BPS působí prostřednictvím více buněčných drah, neomezujících se na estrogenní a androgenní dráhu. Da Silva *et al*. (2019) popsali změnu hladin thyroxinu a trijodthyroninu v plazmě při expozici jedinců BPS. Thyroxin je hormon štítné žlázy, který mimo jiné u samic v období březosti reguluje genovou expresi a zároveň má během časného vývoje mozku plodu vliv na proteiny podílející se na pozdější diferenciaci mozku. V souvislosti s tím, Qui *et al*. (2016) ve své studii popsali, že thyroxin částečně zprostředkovává (oslabuje) stimulační účinky BPS na reprodukční neuroendokrinní systém a s tím související expresi genů v prenatálním vývoji jedinců.

Změna steroidogeneze vlivem přítomnosti bisfenolu S ve folikulární tekutině byla následně prokázána i ve studiích využívajících bovinní granulózní buňky (Campen *et al*., 2018) a granulózní buňky žen, podstupujících *in vitro* fertilizaci (Amar *et al*., 2020).

Funkční interakce mezi drahami aktivovanými estrogenovými, thyroidními a aromatázovými receptory může sloužit jako důležitá regulační vazba v endokrinních systémech. Všechny tyto dráhy jsou zároveň nezbytné k pozorování plných účinků BPS na změny exprese genů v reprodukčním neuroendokrinním systému.

Eladak *et al*. (2015) popsali negativní účinek BPS na testikulární tkáň a oxidativní stres samců potkanů. Zdá se, že expozice BPS nejen indukuje oxidační stres, ale také zvyšuje aktivitu antioxidačních enzymů v tkáni. Se zvyšující se dávkou BPS dochází k výraznému zvýšení testikulárních reaktivních druhů kyslíku a lipidové peroxidace, zatímco aktivita antioxidačních enzymů, obsah proteinů, koncentrace plazmatu a intratestistického testosteronu jsou významně sníženy. Tyto údaje naznačují, že BPS má potenciál indukovat oxidační stres ve varlatech a může mít vliv na spermatogenezi u potkanů, jak potvrdily i další studie, zabývající se genotoxickým a cytotoxickým efektem BPS v souvislosti s posílením vzniku reaktivních forem kyslíku (George and Rupasinghe, 2018; Hercog *et al*., 2019).

Negativní vliv expozice BPS byl prokázán i ve spojení s utvářením mléčných žlaz (Kolla *et al*., 2018) a tělesným vývojem (Ahsan *et al*., 2018) samic potkanů. Kolla *et al*. (2018) popsali vliv BPS na mléčné žlázy samic myší, jejichž utváření je v průběhu života silně ovlivňováno hormony. V závislosti na věku samic a dávce BPS byly pozorovány specifické estrogenní účinky BPS, zcela odlišné od účinků jiných xenoestrogenů. Prenatální expozice samic vede k tvorbě intraduktální hyperplazie mléčných žlaz v dospělosti. Prepubertání expozice pak vede k pozměněné morfologii mléčných žlaz, opožděnému nástupu puberty, zvýšení tělesné hmotnosti, snížené hmotnosti dělohy a změněným hladinám plazmatických hormonů. Zaznamenána byla zvýšená acyklie vaječníků spojená se zvýšeným počtem cystických a atretických folikulů a poklesem počtu ovulujících folikulů (Ahsan *et al*., 2017; Kolla *et al*., 2018; Shi *et al*., 2019).

Atlas and Dimitrova (2019) následně prokázali vliv BPS na organizaci epiteliárních buněk mléčné žlázy žen, kde BPS způsobil narušení organizace buněk a nadměrnou expresi onkogenů. Potvrdili tedy jeho vliv na vývoj mléčné žlázy a přispění k rozvoji rakoviny prsu.

Navíc, Grandin *et al*. (2019) prokázali nízký materno – fetální prostup metabolitů BPS placentou v průběhu těhotenství z krevního oběhu matky.

V dalších experimentech zaměřených na buněčné kultury bylo prokázáno, že BPS působí cytotoxicky, genotoxicky (Lee *et al*., 2013) a mutagenně (Fic *et al*., 2013) tím, že indukuje chromozomové aberace a výrazná poškození DNA i přes samovolný proces homologní rekombinace. Důvodem těchto negativních účinků může být vazba na sérové albuminy nebo poškození DNA a následné ovlivnění několika signálních kaskád kdekoliv uvnitř organismu (Lee *et al*., 2013; Mathew *et al*., 2014), včetně narušení buněčné signalizace v apoptotických drahách (Salvesen and Walsh, 2014).

Negativní vliv BPS na reprodukci samic savců byl prokázán i naším týmem, který již publikoval část výsledků, na které navazují výsledky této práce (Zalmanova *et al*., 2017; Nevoral *et al*., 2018).

Díky *in vitro* expozici prasečích oocytů BPS v průběhu meiotickému zrání, dochází k selhání tvorby tubulínových vláken, které jsou součástí meiotického vřetene a kontrolují správnou segregaci chromozomů. Dále byly prokázány změny v zásobování maternální mRNA, která je nezbytná pro úspěšný vývoj oocytu a embrya v době zrání a po oplození, než je zahájena transkripce embryonální mRNA. Zároveň byly potvrzeny změny v množství proteinů a distribuci estrogenových receptorů α a β a aromatázy, jak bylo popsáno v dřívějších studiích u ryb a somatických buněk savců. Tato studie vlivu BPS na meiotické zrání oocytů prasat byla posléze doplněna obdobnými výsledky u bovinních oocytů (Campen *et al*., 2018).

Ve studii Nevoral *et al*. (2018), ve které byl využit myší experimentální model, byly samicím podávány *in vivo* nízké koncentrace BPS simulující reálný výskyt tohoto ED v životním prostředí. U skupiny myší krmených stravou s přidaným BPS došlo k redukci hmotnosti ovarií a počtu primárních a preantrálních folikulů. Dále byl zaznamenán zvýšený objem antrálních folikulů, což může být vysvětleno kompenzací redukce počtu folikulů. Ve stejné studii byly analyzovány potenciální epigenetické změny, reprezentované změnou methylace DNA, přesněji 5 methyl – cytosinu (5mC) a dimethylace lysinu K27 na histonu H3 (H3K27me2). Byly zaznamenány změny v methylaci H3K27me2 u oocytů vystavených různým

koncentracím BPS. Toto zjištění demonstruje obdobnou epigenetickou aktivitu, která byla zaznamenána u BPA (Nevoral *et al*., 2018).

Náhrada BPA za BPS se díky nedostatečnému prostudování jevila jako optimální. Nyní se však zdá, že je tomu přesně naopak. Nově získávané poznatky tedy stále jasněji ukazují, že BPS není zdaleka tak bezpečná látka, za jakou byla považována.

3. Hypotéza

Na základě dostupných informací byla stanovena hypotéza, že BPS negativně ovlivňuje průběh meiotického zrání savčích oocytů, epigenetický vzor oocytů a utváření cytoskeletárních struktur oocytů.

4. Cíle práce

Za účelem ověření stanovené hypotézy byly navrženy následující cíle:

1.) prokázat vliv BPS na průběh meiotického zrání

2.) sledovat změny v expresi a buněčné lokalizaci proteinových markerů meiotického zrání

3.) sledovat změny v distribuci vybraných markerů meiotického zrání – laminu A / C, α – tubulinu, methylaci DNA a histonu K3H27

5. Materiály a metodika

5.1. Experimentální model prase (*Sus scrofa*)

5.1.1. Etické zásady

Všechny experimenty byly provedeny v souladu se stávajícími zákony České republiky a všechny experimentální protokoly byly schváleny etickou komisí České zemědělské univerzity v Praze.

5.1.2. Chemikálie

Všechny použité chemikálie byly zakoupeny u společnosti Sigma Aldrich (St. Louis, MO, USA), pokud není uvedeno jinak.

5.1.3. Získávání a kultivace prasečích oocytů

Vaječníky prasat byly získávány z jatečně poražených prepubertálních prasniček. Kumulo – oocytární komplexy (COCs) byly z vaječníků získávány aspirací folikulární tekutiny z folikulů o velikosti 2 – 5 mm pomocí injekční stříkačky s jehlou 18G. Pod stereomikroskopem byly z folikulární tekutiny vybírány pouze COCs s neporušenou cytoplazmou a kompaktním kumulárním obalem.

Před samotnou kultivací byly vybrané COCs nejdříve třikrát promyty v kultivačním médiu bez přídavku BPS a posléze rozděleny do jedné kontrolní (K) a třech pokusných skupin (3nM, 300nM, 30µM). Kultivace probíhala ve čtyřjamkové destičce 4 – well multidish (Nunc, Denmark), v 1 ml modifikovaného kultivačního média MI99 obohaceného o hydrogen uhličitan sodný (0,039 ml 7 % roztoku na 1 ml média), laktát vápenatý (0,6 mg / ml), gentamicin (0,025 mg / ml), HEPES (1,5 mg / ml), gonadotropní hormony eCG a hCG v poměru 13,5 I.U : 6,6 I.U. / ml (P.G.600, Intervet, Boxmeer, Holland) a folikulární tekutinu (100 µl / ml), v podmínkách řízené atmosféry o obsahu 5 % CO2 ve směsi se vzduchem při 39°C. Do kultivačního média pokusných skupin byl přidán roztok BPS o výsledné koncentraci 3nM, 300nM a 30µM BPS. Kultivace probíhala po dobu 16, 18, 20, 22, 24 a 48 hodin.

5.1.4. Ošetření oocytů

Po celou dobu kultivace byly COCs pokusných skupin v kultivačním médiu za přítomnosti roztoku BPS. BPS rozpuštěný v DMSO byl přidán do kultivačního média, čímž bylo docíleno finální koncentrace 3 nM, 300 nM a 30 µM BPS. Finální koncentrace DMSO byla vždy 0,1 %. Oocyty kontrolní skupiny byly ošetřeny pouze 0,1 % DMSO.

5.1.5. Morfologické hodnocení stádií GVBD

COCs byly po příslušné době kultivace zbaveny kumulárních buněk pipetováním pomocí tenkostěnné kapiláry. Poté byly oocyty:

- fixovány v 4 % roztoku paraformaldehydu v PBS (Phosphate buffered saline), při laboratorní teplotě po dobu půl hodiny, permeabilizovány a blokovány v 0,1 % Tritonu X – 100 (v 1 % a 5 % kozím séru ve fosfátovém pufru, NGS – PBS) a následně podrobeny imunocytochemickému barvení (popsáno v 5.1.6.) – platí pro oocyty kultivované 16, 18, 20 a 22 hodin.
- montovány na podložní skla, fixovány po dobu nejméně 24 hod. v roztoku ethanol : kyselina octová (3 : 1) a následně barveny 1 % orceinem a vyhodnoceny pod mikroskopem s fázovým kontrastem (zvětšení 400x) – platí pro oocyty kultivované 24 a 48 hodin.

Na základě délky kultivace a dosaženého stádia jaderného zrání byly oocyty rozděleny na: rozpad zárodečného váčku (GVBD) ve stádiu GV1, GV2, GV3 a GV4, přechod mezi prometafází a metafází I (16 – 22h kultivace); pozdní diakineze; přechod mezi anafází I a telofází I; metafáze I (24h kultivace) a metafáze II (48h kultivace) (Motlik and Fulka, 1976).

5.1.6. Lokalizace vybraných proteinových struktur

Po ukončení příslušné doby kultivace byly oocyty zbavené kumulárních buněk fixovány v 4 % roztoku paraformaldehydu v PBS při laboratorní teplotě po dobu půl hodiny a skladovány ve 4°C v roztoku PBS s azidem až do dalšího využití. Pro další využití byly oocyty permeabilizovány a blokovány v 0,1 % Tritonu X – 100 (v 1 % a 5 % kozím séru ve fosfátovém pufru, NGS – PBS). Poté byly oocyty inkubovány s primární monoklonální protilátkou

specifickou pro daný protein (anti – lamin A / C; Abcam, UK) po dobu 1 hodiny (1:200, při 39°C, 5 % CO2) v 1 % NGS. Po uplynutí doby kultivace byly oocyty 3x promyty v roztoku 1 % NGS a následně kultivovány se sekundární kozí anti – myší IgG protilátkou konjugovanou s fluoresceiny FITC (fluorescein isothiokyanát; 1:200). Inkubace se sekundární protilátkou probíhala při laboratorní teplotě v 1 % NGS po dobu 40 minut. Po inkubaci byly oocyty 3x propláchnuty v 1 % NGS a namontovány na sklo s kapkou Vectashield obsahující 4'6 – diamidino – 2 – fenylindol (DAPI; Thermo Fisher Scientific) pro obarvení chromatinu.

5.1.7. Statistická analýza

Data jsou prezentována jako průměr ± SEM z nejméně tří nezávislých experimentů, minimální počet oocytů na skupinu je 45. Pro statistickou analýzu dat byl použit neparametrický F – test a Shapiro – Wilkův test normality. Statistická analýza byla prováděna v programu 9.3 SAS (SAS Institute Inc., Cary, NC, USA). P hodnota menší než 0,05 byla považována za statisticky významnou.

5.1.8. Experimentální schéma

Hodnocení vlivu BPS na průběh meiotického zrání oocytů

Vliv BPS na jaderné zrání oocytů v průběhu GVBD

V experimentu byl zjišťován vliv jednotlivých koncentrací BPS (3nM, 300nM, 30µM) na průběh GVBD prasečích oocytů *in vitro*. Oocyty byly kultivovány v podmínkách *in vitro* 16, 18, 20 a 22 hodin za přítomnosti BPS, pro zjištění vlivu BPS na dosažení jednotlivých fází GVBD. Podle morfologického hodnocení chromatinu oocytů byla stanovena fáze GVBD oocytů (GV1, GV2, GV3, GV4).

Vliv BPS na jaderné zrání oocytů v průběhu MI a MII

V experimentu byl zjišťován vliv jednotlivých koncentrací BPS (3nM, 300nM, 30µM) na průběh meiotického zrání prasečích oocytů *in vitro*. Oocyty byly kultivovány v podmínkách *in vitro* 24 hodin (MI) a 48 hodin (MII) za přítomnosti BPS, pro zjištění vlivu BPS na jednotlivé

fáze meiotického zrání. Podle morfologického hodnocení chromatinu oocytů byla stanovena fáze meiotického zrání oocytů.

Hodnocení vlivu BPS na lokalizaci LMNA v průběhu meiotického zrání

Vliv BPS na lokalizaci LMNA v průběhu GVBD

V experimentu byl sledován vliv jednotlivých koncentrací BPS (3 nM, 300 nM, 30 µM) na lokalizaci LMNA v průběhu GVBD prasečích oocytů *in vitro*. Oocyty byly kultivovány v podmínkách *in vitro* 16, 18, 20 a 22 hodin za přítomnosti BPS, pro zjištění vlivu BPS na lokalizaci LMNA v jednotlivých fázích GVBD.

Vliv BPS na lokalizaci LMNA v průběhu MI a MII

V experimentu byl sledován vliv jednotlivých koncentrací BPS na lokalizaci LMNA v průběhu meiotického zrání oocytů *in vitro*. Oocyty byly kultivovány v podmínkách *in vitro* 24 (MI) a 48 hodin (MII) za přítomnosti BPS, pro zjištění vlivu BPS na lokalizaci LMNA v průběhu dosažení kontrolních fází meiotického zrání.

5.2. Experimentální model myš (*Mus musculus*)

5.2.1. Etické zásady

Všechny experimenty byly provedeny v souladu se zákonem č. 246 / 1992 Sb. o ochraně zvířat proti týrání pod dohledem Poradního výboru pro dobré životní podmínky zvířat na Ministerstvu školství, mládeže a tělovýchovy České republiky, ID schválení MSMT – 11925 / 2016 – 3. Všechny experimentální protokoly byly schváleny etickou komisí Karlovy Univerzity v Praze.

5.2.2. Chemikálie

Všechny použité chemikálie byly zakoupeny u společnosti Sigma Aldrich (St. Louis, MO, USA), pokud není uvedeno jinak.

5.2.3. Využitý experimentální kmen a chovné podmínky

Samice ICR (outbrední kmen) myší ve věku od šesti do sedmi týdnů byly zakoupeny od společnosti Velaz Ltd. (Česká republika), chovány v intaktních polysulfonátových klecích a udržovány v podmínkách s 12 – ti hodinovým cyklem světlo / tma, při teplotě 21 ± 1 ° C a relativní vlhkosti 60 %. Dieta neobsahující fytoestrogeny (1814 P; Altromin, Německo) a ultračistá voda (ve skleněných lahvích, měněna dvakrát týdně) byly poskytovány *ad libitum*. Zvířata byla aklimatizována alespoň jeden týden před zahájením experimentů.

5.2.4. Expoziční schéma

Zvířata (N = 75) byla náhodně rozdělena do pěti experimentálních skupin po 15 – ti zvířatech / skupina, které zahrnovaly jednu kontrolní skupinu a čtyři skupiny ošetřené jednou ze čtyř vybraných dávek BPS (0,001; 0,1; 10 a 100 ng BPS x g tělesné váhy^{-1} den^{-1}, dále označovaných jako BPS1, BPS2, BPS3, respektive BPS4). Pokusy byly prováděny v pěti nezávislých opakováních. BPS byl rozpuštěn v 50 µl 50 % glycerolu obsahujícího 0,1 % dimethylsulfoxidu a podáván denně po dobu sedmi dnů orální sondou. Po expozici byly myši usmrceny cervikální dislokací a jejich vaječníky byly odebrány pro další experimenty.

5.2.5. Získávání a kultivace myších oocytů

Pro získání plně dorostlých, nezralých oocytů ve stádiu GV, byly na odebraných ováriích pomocí jehly 27G narušeny ovariální folikuly. Získané GV oocyty byly následně manipulovány v médiu M2 doplněném o 100 µM isobutyl – methylxanthinu (IBMX), specifickým inhibitorem endogenní fosfodiesterázy, za účelem zachování intaktních GV oocytů (Grondahl *et al*., 1998). Plně dorostlé intaktní GV oocyty byly následně, po ukončení manipulace, umístěny do kultivačního média M16 s IBMX, po dobu alespoň 1 h při 37 ° C a 5 % CO2, pro regeneraci oocytárních proteinů. Poté byly oocyty fixovány ve 4 % paraformaldehydu ve fosfátovém pufrovacím solném roztoku (PBS), doplněny 0,1 % polyvinylalkoholem (PVA), po dobu 30 minut při pokojové teplotě (22° C) a uloženy při 4° C pro další použití. Alternativně byly získané GV oocyty kultivovány v kultivačním médiu M16 bez IBMX po dobu 16 hodin při 37 ° C a 5 % CO2, pro zisk maturovaných MII oocytů. Zralé oocyty s vyloučenými pólovými tělísky byly fixovány a uloženy, jak je popsáno výše.

5.2.6. Terminální deoxynukleotidyl – transferázové dUTP značení a barvení (TUNEL)

Fixované MII oocyty byly permeabilizovány v 0,1 % Tritonu X – 100 v PBS obsahujícím 0,05 % NaN_3 po dobu 40 minut. Oocyty byly ošetřeny dUTP konjugovaným s fluoresceinem a terminálním deoxyribonukleotidyl – transferázovým enzymem (In situ Cell Death Detection Kit, kat. č. 11684795910, Roche, Německo) po dobu 1 hodiny ve tmě při 37°C. Pozitivní kontrola byla připravena pomocí soupravy DNase I (AMP – D1, Sigma – Aldrich). Nakonec byly oocyty namontovány na sklíčka s médiem Vectashield DAPI (4', 6 – diamidino – 2 – fenylindol; VectorLaboratories Inc., USA). Intenzita signálu byla měřena pomocí softwaru ImageJ (National Institutes of Health, USA).

5.2.7. Lokalizace vybraných proteinových struktur

Fixované oocyty byly permeabilizovány v PBS s přídavkem 0,04 % Triton X – 100 a 0,3 % Tween – 20 po dobu 15 minut. Byly hodnoceny heterochromatinové markery oocytů, včetně 5' – methylcytosinu (5meC) a dimethylace histonu H3 na lysinu K27 (H3K27me2). Kolokalizace 5meC a H3K27me2 byla provedena pomocí HCI a trypsinu, jak bylo popsáno dříve (Santos *et al*., 2002). Poté byly oocyty blokovány v 1 % roztoku bovinního sérového albuminu (BSA) v PBS s Tween – 20 po dobu 15 minut a inkubovány s protilátkami anti – α – tubulinem (1: 200, Sigma – Aldrich), anti – 5meC (1: 200, Sigma – Aldrich) a anti – H3K27me2 (1: 200, Abcam, UK). Po promytí byly oocyty inkubovány s protilátkami anti – myším a anti – králičím AlexaFluor 488 a 647 (1: 200). Phalloidin (1: 200; Thermo Fisher Scientific, USA) byl přidán do promývání a použit pro vizualizaci β – aktinu. Obarvené oocyty byly namontovány na sklíčka v médiu Vectashield DAPI. Intenzita signálu byla měřena pomocí softwaru ImageJ. Byla také hodnocena konfigurace chromatinu kolem jádra [SN, NSN (Zuccotti *et al*., 1998)] a vyloučení pólového tělíska. Snímky byly získány za použití konfokálního mikroskopu s rotujícím diskem Olympus IX83 (Olympus, Německo) a softwaru VisiView (Visitron Systems GmbH, Německo).

5.2.8. Statistická analýza

Data byla zpracována pomocí programu Statistica Cz 12 (StatSoft, Inc., USA). Statistický test Kruskal – Wallis ANOVA byl použit pro kvantitativní proměnné a χ^2 – kvadrát

test byl použit pro proporční testování. Významné rozdíly mezi jednotlivými páry skupin byly hodnoceny *post hoc* pomocí vícenásobného srovnání průměrných řad, Mann – Whitney U test s Bonferroniho korekcí, nebo Fisherův exaktní test s Bonferroniho korekcí. Kde to bylo vhodné, byly korelace mezi proměnnými hodnoceny pomocí Spearmanovy metody. Statistický význam byl nastaven na $\alpha = 0,05$ a všechny použité hodnoty P a testy byly oboustranné.

5.2.9. Experimentální schéma

Hodnocení vlivu BPS na vybrané markery před zahájením meiotického zrání

Vliv BPS na kvantitu a meiotickou způsobilost GV oocytů

V experimentu byl zjišťován vliv jednotlivých koncentrací BPS (0,001; 0,1; 10 a 100 ng BPS x g tělesné váhy^{-1} den^{-1}) na kvantitativní a kvalitativní markery GV oocytů myší před zahájením meiotického zrání *in vitro*. GV oocyty byly získány z myší, které byly po dobu jednoho týdne vystaveny různým koncentracím BPS *in vivo* pro zjištění vlivu BPS na výtěžnost a meiotickou způsobilost získaných GV oocytů. Podle morfologického hodnocení chromatinu byla stanovena fáze GV oocytů (NSN, SN).

Vliv BPS na změnu v methylaci DNA a histonu H3K27 u GV oocytů

V experimentu byl zjišťován vliv jednotlivých koncentrací BPS (0,001; 0,1; 10 a 100 ng BPS x g tělesné váhy^{-1} den^{-1}) na vybrané změny v epigenomu GV oocytů myší před zahájením meiotického zrání *in vitro*. GV oocyty byly získány z myší, které byly po dobu jednoho týdne vystaveny různým koncentracím BPS *in vivo,* pro zjištění vlivu BPS na změnu v epigenetickém vzoru DNA a histonu H3K27 získaných GV oocytů.

Hodnocení vlivu BPS na vybrané markery v průběhu meiotického zrání

Vliv BPS na formaci dělícího vřetene u MII oocytů

V experimentu byl zjišťován vliv jednotlivých koncentrací BPS (0,001; 0,1; 10 a 100 ng BPS x g tělesné váhy^{-1} den^{-1}) na utváření meiotického vřetene v průběhu meiotického zrání *in vitro*. Myši byly po dobu jednoho týdne vystaveny různým koncentracím BPS *in vivo*, po ukončení expozice byly odebrané oocyty kultivovány v podmínkách *in vitro* 16 hodin (MII)

bez přítomnosti BPS, pro zjištění vlivu BPS na formaci tubulárních vláken v průběhu meiotického zrání.

Vliv BPS na integritu DNA u MII oocytů

V experimentu byl zjišťován vliv jednotlivých koncentrací BPS (0,001; 0,1; 10 a 100 ng BPS x g tělesné váhy^{-1} den^{-1}) na změny v integritě DNA MII oocytů v průběhu meiotického zrání *in vitro*. Myši byly po dobu jednoho týdne vystaveny různým koncentracím BPS *in vivo*, po ukončení expozice byly odebrané oocyty kultivovány v podmínkách *in vitro* 16 hodin (MII) bez přítomnosti BPS, pro zjištění vlivu BPS na změnu ve výskytu dvojitých zlomů DNA v průběhu meiotického zrání.

Vliv BPS na změnu v methylaci DNA a histonu H3K27 u MII oocytů

V experimentu byl zjišťován vliv jednotlivých koncentrací BPS (0,001; 0,1; 10 a 100 ng BPS x g tělesné váhy^{-1} den^{-1}) na vybrané změny v epigenomu MII oocytů v průběhu meiotického zrání *in vitro*. Myši byly po dobu jednoho týdne vystaveny různým koncentracím BPS *in vivo*, po ukončení expozice byly odebrané oocyty kultivovány v podmínkách *in vitro* 16 hodin (MII) bez přítomnosti BPS, pro zjištění vlivu BPS na změnu v epigenetickém vzoru nukleozómů v průběhu meiotického zrání.

6. Výsledky

6.1. Experimentální model prase (*Sus scrofa*)

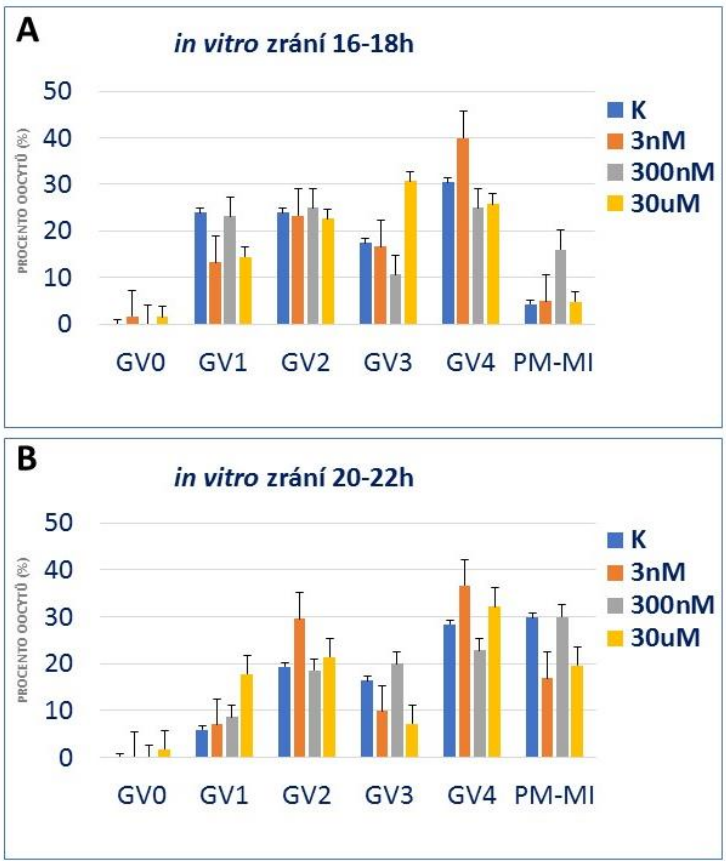
6.1.1. Hodnocení vlivu BPS na průběh meiotického zrání

Vliv BPS na jaderné zrání oocytů v průběhu GVBD

Cílem bylo zhodnotit vliv BPS na průběh jednotlivých fází GVBD. Ačkoliv nebyl pozorován statisticky významný vliv přítomnosti BPS na rychlost průběhu GVBD (Obr. 1A, B). Byly pozorovány tyto trendy: po 16 – 18 hodinách *in vitro* kultivace došlo oproti kontrole u všech pokusných skupin ke zvýšení procenta oocytů, které dosáhly fáze GV3, GV4 a PM – MI (Obr. 1A). U skupiny ošetřené 300 nM BPS došlo k procentuálnímu nárůstu oocytů, které po 16 – 18 hodinách kultivace dosáhly PM – MI, po 20 – 22 hodinách ale byla procenta oocytů,

která dosáhla PM – MI srovnatelná s kontrolou. U koncentrací 3 nM a 300 nM také došlo po 20 – 22 hodinách kultivace ke snížení procenta oocytů, které dosáhly PM – MI (Obr. 1B). Tento trend napovídá počátečnímu zrychlení průběhu GVBD, které je následované postupným zpomalením, u koncentrace 300 nM došlo navíc pravděpodobně k částečné stagnaci GVBD ve stádiu PM – MI po 16 – 18 hodinách kultivace.

Obrázek 1

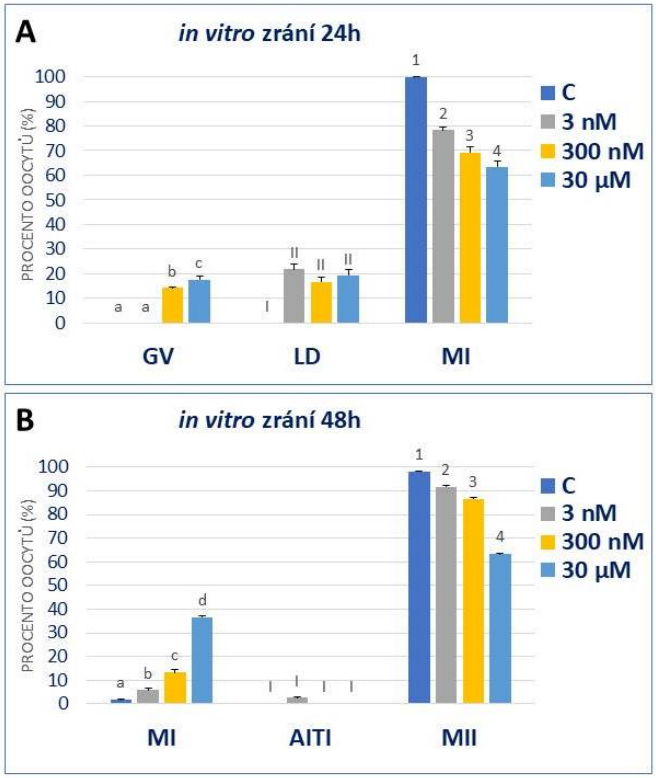


Účinky BPS na schopnost oocytů dosáhnout jednotlivých fází GVBD. (A) Účinky BPS (3 nM, 300 nM a 30 uM) na stupně GVBD dosažených oocyty kultivovanými 16 – 18 h in vitro, (B) Účinky BPS na stupně GVBD dosažených oocyty kultivovanými 20 – 22 h in vitro. K – kontrolní skupina oocytů kultivovaných in vitro bez přítomnosti BPS, GV0 – germinal vesicle 0 (zárodečný váček ve stádiu 0), GV1 – germinal vesicle 1 (zárodečný váček ve stádiu 1),GV2 – germinal vesicle 2 (zárodečný váček ve stádiu 2), GV3 – germinal vesicle 3 (zárodečný váček ve stádiu 3), GV4 – germinal vesicle 4 (zárodečný váček ve stádiu 4), PM – MI prometafáze – metafáze I. Data jsou vyjádřena jako průměr ± SEM ze tří nezávislých experimentů, v každém experimentu bylo sledováno minimálně 45 oocytů na skupinu.

Vliv BPS na jaderné zrání oocytů v průběhu MI a MII

Cílem bylo zhodnotit vliv BPS na jaderné zrání prasečích oocytů po 24 h a 48 h kultivace *in vitro*. Bylo zjištěno, že oocyty, které byly ošetřeny různými koncentracemi BPS (3 nM, 300 nM nebo 30 µM), vykazovaly signifikantní, na dávce závislé snížení schopnosti dosáhnout MI a MII po 24 a 48 hodinách *in vitro* kultivace. Oocyty kultivované s BPS (300 nM a 30 uM) neobnovily meiózu po 24 hodinách *in vitro* kultivace. Avšak po 48 hodinách *in vitro* kultivace, všechny oocyty kultivované s BPS iniciovaly meiotické zrání a dosáhly alespoň stádia MI (Obr. 2A, B).

Obrázek 2



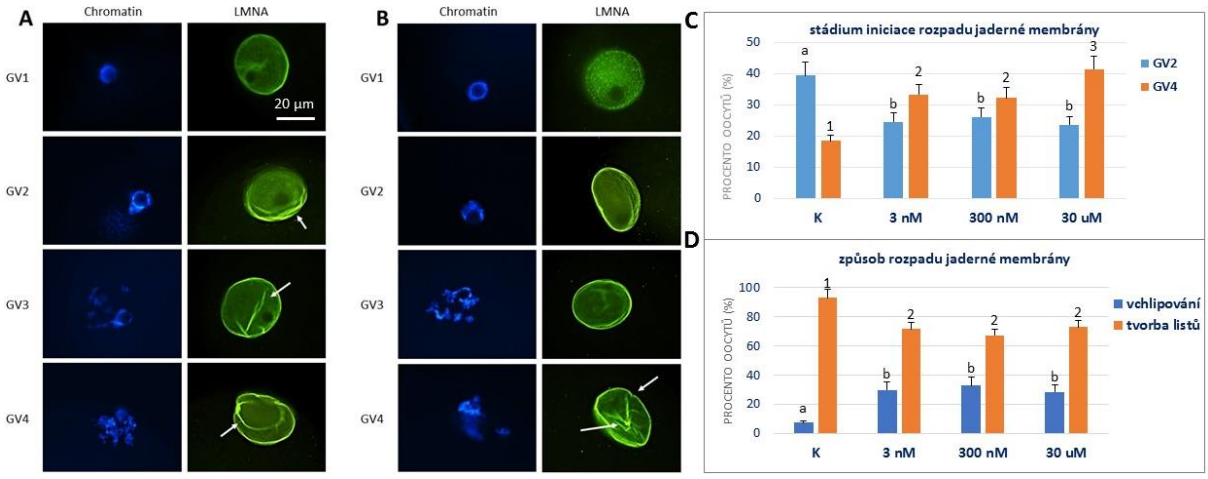
Účinky BPS na meiotické zrání oocytů. (A) Účinky BPS (3 nM, 300 nM a 30 uM) na stupně meiotického zrání dosažených oocyty kultivovanými 24 h, (B) 48 h in vitro. K – kontrolní skupina oocytů kultivovaných in vitro bez přítomnosti BPS, GV – germinal vesicle (zárodečný váček), LD – pozdní diakineze, MI – metafáze I, AITI – anafáze I – telofáze I, MII – metafáze II. Data jsou vyjádřena jako průměr ± SEM *ze čtyř nezávislých experimentů, v každém experimentu bylo sledováno minimálně 120 oocytů na skupinu. Různé horní indexy označují statistický význam při P <0,05.*

6.1.2. Hodnocení vlivu BPS na lokalizaci LMNA v průběhu meiotického zrání

Vliv BPS na lokalizaci LMNA v průběhu GVBD

Cílem bylo zhodnotit vliv přítomnosti BPS na změny v lokalizaci LMNA, jako markeru jaderné membrány, v jednotlivých fázích GVBD. Bylo zjištěno, že oocyty, které byly ošetřeny různými koncentracemi BPS (3 nM, 300 nM, 30 µM), vykazovaly změny v zahájení GVBD, oproti oocytům v kontrolní skupině, kultivovaným bez přítomnosti BPS (Obr. 3A, B). Zatímco u kontrolních oocytů byla iniciace kolapsu jaderné membrány viditelná již od stádia GV2, v pokusných skupinách byl významný nárůst počtu oocytů, které vykazovaly počáteční kolaps mebrány, zjištěn až ve stádiu GV4. Nejvyššího efektu bylo dosaženo u koncentrace 30 µM BPS (Obr. 3C). Zároveň byl u oocytů ošetřených BPS viditelný statisticky významný rozdíl ve způsobu kolapsu jaderné membrány. U kontrolní skupiny docházelo k postupnému překládání jaderné membrány a tvorbě listů (Obr. 3A – bílé šipky; 3D), zatímco u pokusných skupin bylo u významného procenta oocytů viditelné vchlipování membrány směrem do nukleoplazmatického prostoru (Obr. 3B – bílé šipky; 3D).

Obrázek 3



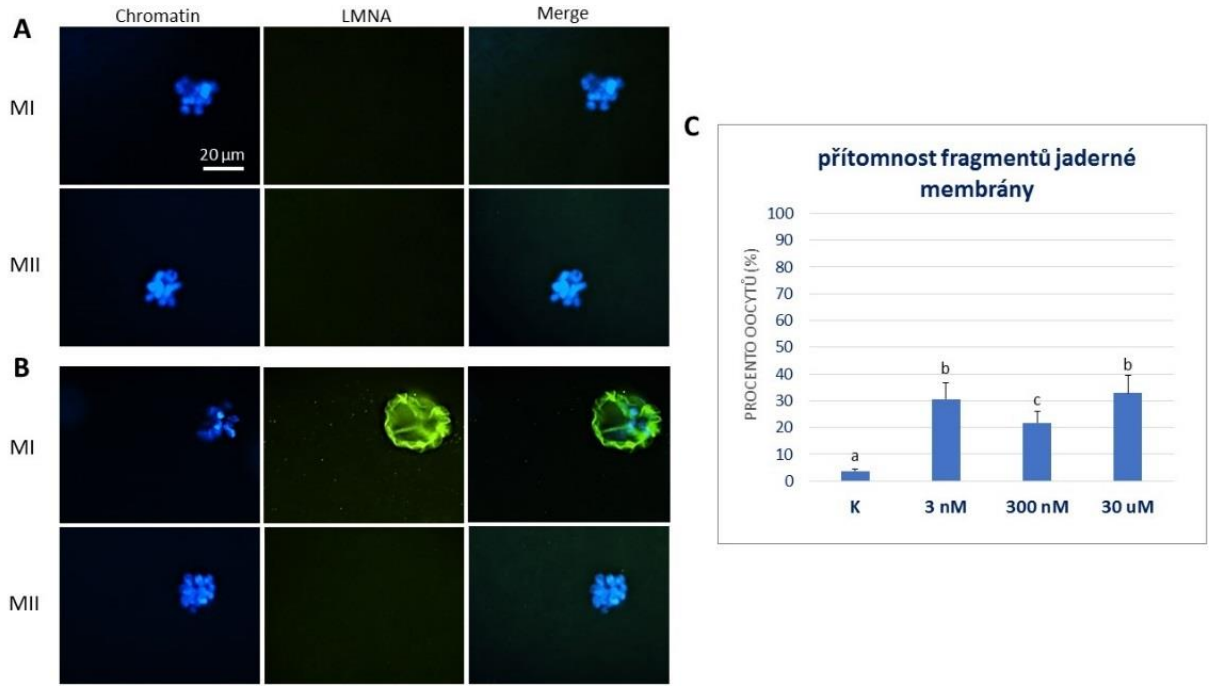
Lokalizace LMNA v průběhu GVBD. (A) Reprezentativní obrázky zobrazují iniciaci rozpadu jaderné membrány u oocytů kontrolní skupiny (GV1 – GV4), kultivovaných 16 – 22 h in vitro. (B) Reprezentativní obrázky zobrazující zpoždění iniciace GVBD u oocytů pokusných skupin (GV1 – GV4) kultivovaných 16 – 22 h in vitro za přítomnosti BPS. Rozdílný způsob kolapsu membrány u oocytů kontrolní skupiny (A) a oocytů pokusných skupin (B) označují bílé šipky. Zelená barva označuje LMNA, modrá označuje chromatin. (C) Graf závislosti

koncentrace BPS na stádium iniciace GVBD. (D) Graf závislosti koncentrace BPS na způsob GVBD. Data jsou vyjádřena jako průměr ± SEM ze tří nezávislých experimentů, v každém experimentu bylo sledováno minimálně 65 oocytů na skupinu. Různé horní indexy označují statistickou signifikanci při P <0,05 a P <0,01.

Vliv BPS na lokalizaci LMNA v průběhu MI a MII

Cílem bylo zhodnotit vliv BPS na lokalizaci LMNA v průběhu MI, respektive MII (Obr. 4A, B). U všech pokusných skupin ošetřených BPS bylo pozorováno významné zvýšení počtu oocytů, které si v průběhu MI zachovaly fragmenty jaderné membrány, přičemž nejvyšší statisticky významný rozdíl byl pozorován u oocytů ošetřených 3 nM a 30 µM BPS (Obr. 4C). Toto zjištění koresponduje s předchozími experimenty, ve kterých bylo prokázáno významné zpoždění iniciace rozpadu jaderné membrány v pokusných skupinách, oproti oocytům v kontrolní kupině. V průběhu MII již nebyl LMNA lokalizován v žádné z pokusných, ani v kontrolní skupině (Obr. 4A, B).

Obrázek 4



Lokalizace LMNA v průběhu MI, MII. (A) Reprezentativní obrázky ukazují úplný rozpad jaderné membrány u oocytů kontrolních skupin kultivovaných 24 h (MI) a 48 h (MII) in vitro bez přítomnosti BPS. (B) Dále obrázky ukazují reprezentativní obrázky zachování fragmentů

jaderné membrány po 24 h a úplný rozpad jaderné membrány po 48 h in vitro kultivace za přítomnosti BPS u oocytů pokusných skupin. Zelená barva označuje LMNA, modrá označuje chromatin. (C) Graf závislosti koncentrace BPS na přítomnost fragmentů jaderé membrány ve stádiu MI. Data jsou vyjádřena jako průměr ± SEM tří nezávislých experimentů, v každém experimentu bylo sledováno minimálně 65 oocytů na skupinu. Různé horní indexy označují statistickou signifikanci při P <0,01 a P <0,05.

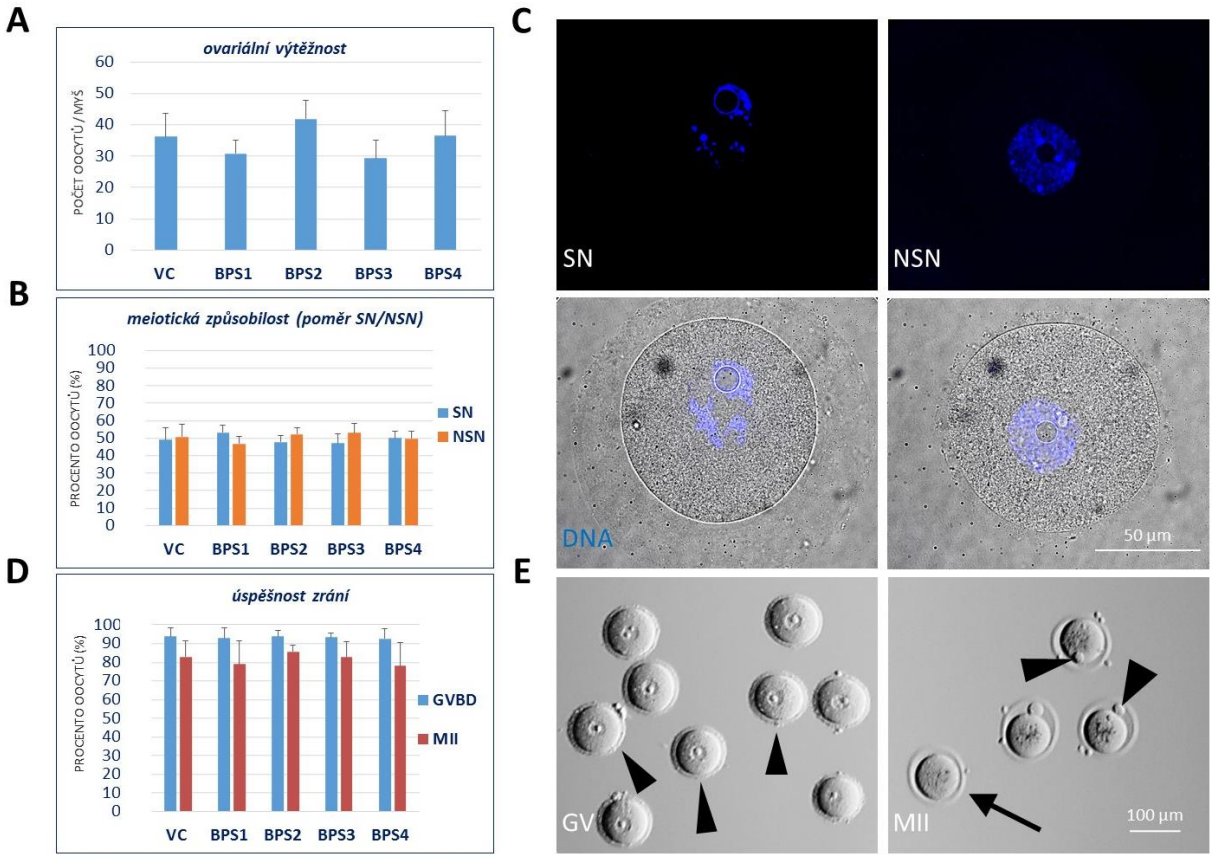
6.2. Experimentální model myš (*Mus musculus*)

6.2.1. Hodnocení vlivu BPS na vybrané markery před zahájením meiotického zrání

Vliv BPS na kvantitu a meiotickou způsobilost GV oocytů

Cílem bylo zhodnotit vliv BPS na ovariální výtěžnost a stádium GV oocytů (SN / NSN), izolovaných z hormonálně nestimulovaných samic myší po *in vivo* expozici BPS. Poměr SN a NSN v GV oocytech a rychlost GVBD / maturace ve zralých oocytech byly analyzovány s použitím fluorescenčního značení chromatinu. Nebyl pozorován statisticky významný vliv BPS na výtěžek GV oocytů (obr. 5A). Podobně, poměr SN / NSN se neodlišoval mezi kontrolní skupinou a skupinami ošetřenými BPS (obr. 5B). Ani míra zrání (obr. 5D) neprokázala statisticky významné rozdíly, což naznačuje, že expozice BPS při testovaných hladinách nemá žádný vliv na celkové množství ani kvalitu oocytů.

Obrázek 5



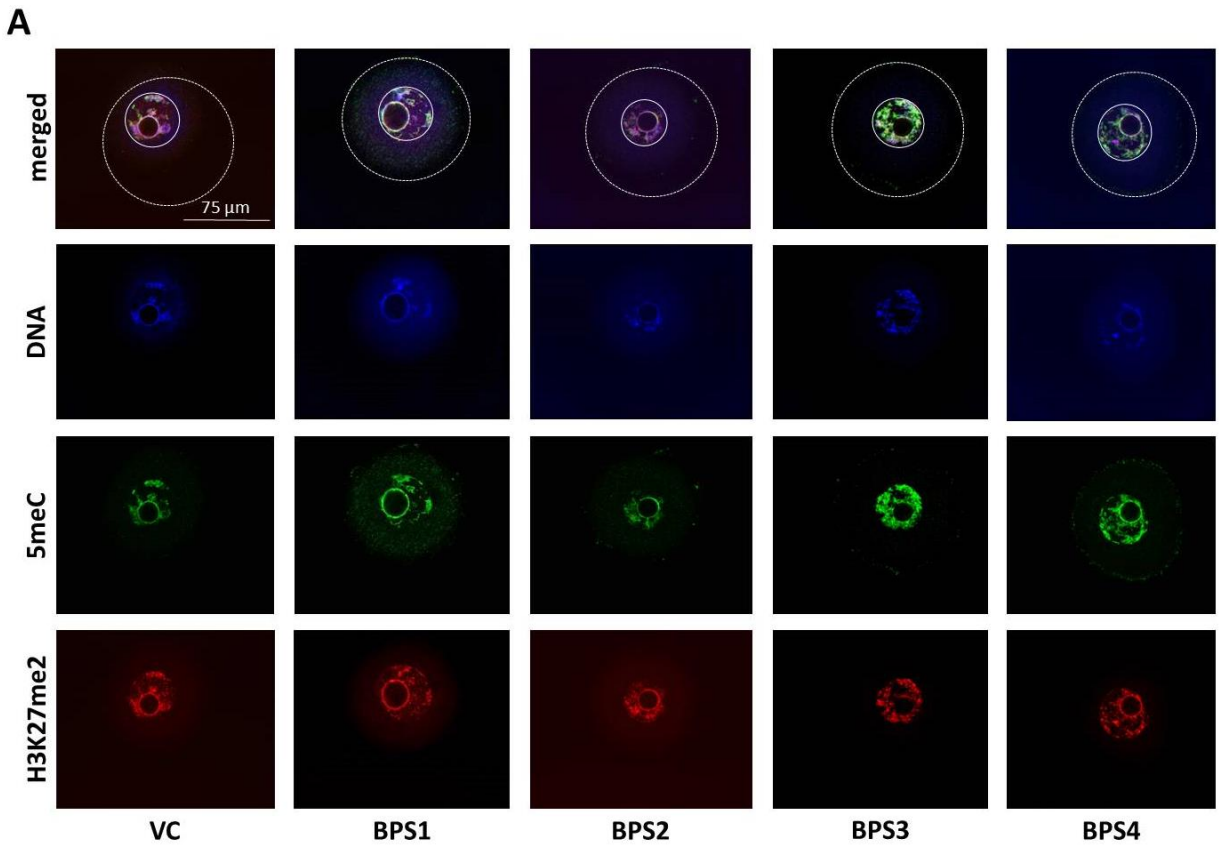
Množství a meiotická způsobilost oocytů ve fázi zárodečného váčku (GV) izolovaných po expozici samic myší BPS. (A) Výtěžek oocytů na základě počtu oocytů izolovaných z jedné samice, N=12 (počty samic ze čtyř nezávislých experimentů). (B) Proporční zobrazení ohraničeného (SN) a neohraničeného (NSN) jadérka, n≥35 (počty oocytů ze šesti nezávislých experimentů). (C) Reprezentativní fluorescenční snímky oocytů SN a NSN ve fázi GV. (D) Rozpad GV (GVBD) a rychlost zrání oocytů, odrážející schopnost GV oocytů znovu zahájit meiózu a dosáhnout stádia MII, n≥261 (počty oocytů ze šesti nezávislých experimentů). (E) Reprezentativní snímky nezralých GV a zralých MII oocytů v procházejícím světle. Šipka označuje oocyt, který prošel GBVD, ale nedosáhl stádia MII. Trojúhelníky označují GV a vydělené pólové tělísko jako markery nezralého GV, respektive zralých MII oocytů. Data jsou vyjádřena jako medián, minimální a maximální hodnoty.

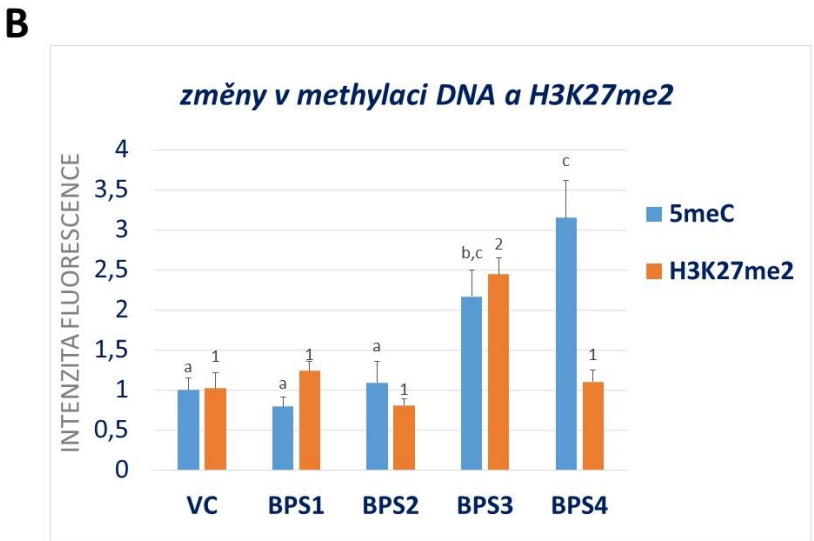
<u>Vliv BPS na změnu v methylaci DNA a histonu H3K27 u GV oocytů</u>

Cílem bylo zhodnotit vliv BPS na epigenetické alterace GV oocytů po ošetření BPS *in vivo.* Jelikož celogenomová methylace DNA a histonu H3 tvoří markery stability

heterochromatinu, byly 5meC a H3K27me2 hodnoceny na úrovni celé buňky pomocí imunocytochemie (obr. 6A). Bylo zjištěno, že hladiny 5meC byly významně zvýšeny ve skupinách BPS3 a BPS4 ve srovnání s hladinou 5meC u skupiny BPS2. Statisticky významný rozdíl však nebyl pozorován ve srovnání s kontrolními oocyty (obr. 6B). Statisticky významně byla oproti kontrole v BPS3 oocytech zvýšena dimethylace H3K27 (obr. 6B).

Obrázek 6





Celogenomové epigenetické markery v nezralých oocytech ve stádiu zárodečného váčku (GV). (A) Reprezentativní snímky 5meC (zelená) a H3K27me2 (červená) represivních markerů chromatinu v nezralých GV oocytech. Přerušované a plné kruhy ukazují hranici oocytů a zárodečného váčku. (B) Integrovaná hustota signálu pro 5meC a H3K27me2 ve srovnání s hustotou kontrolní skupiny. Data jsou vyjádřena jako průměr ± SEM ze tří nezávislých experimentů, v každém experimentu bylo sledováno minimálně 21 oocytů na skupinu. Různé horní indexy označují statistický význam při P <0,05 a P <0,01 nebo P <0,0001.

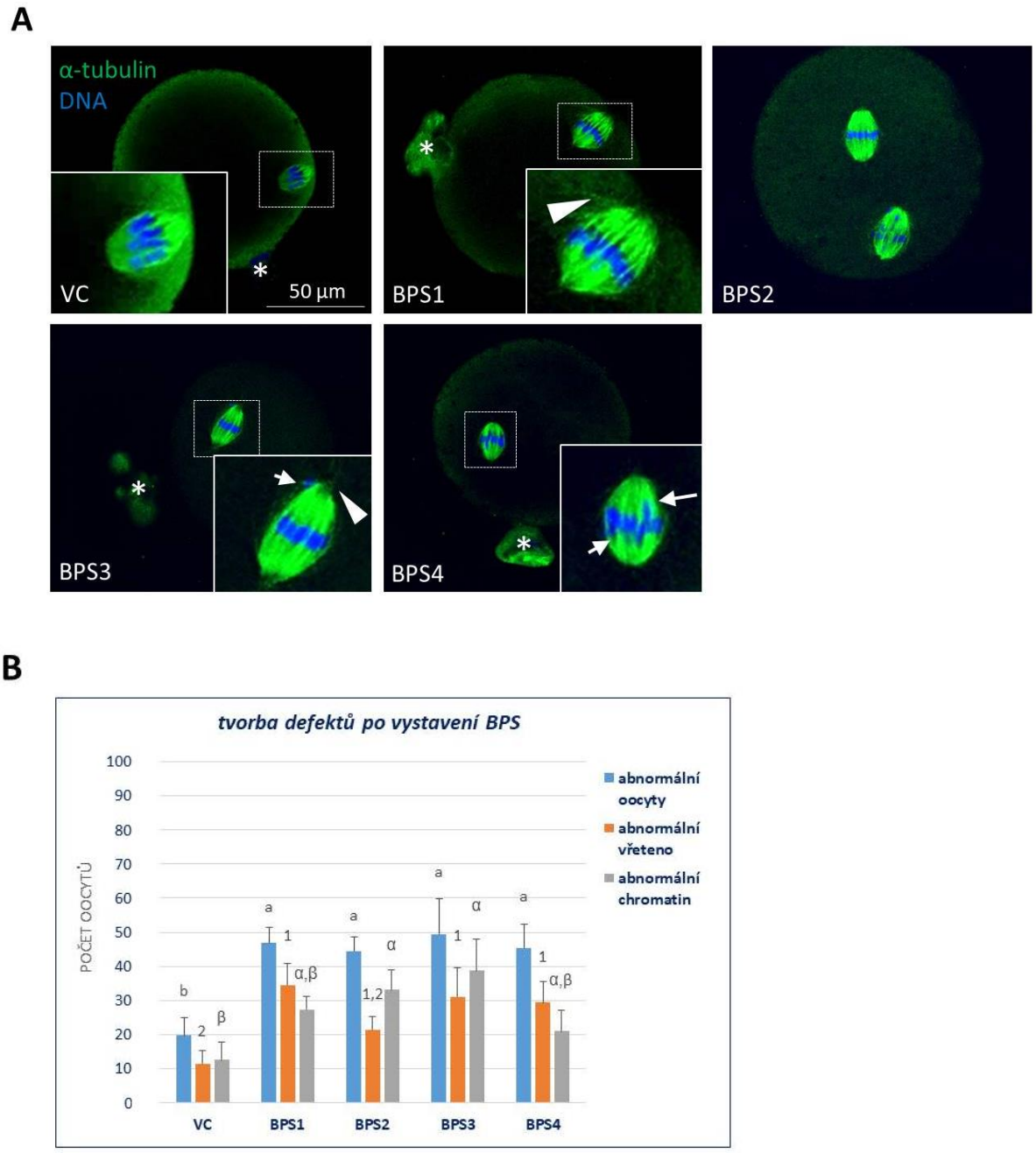
6.2.2. Hodnocení vlivu BPS na vybrané markery v průběhu meiotického zrání

Vliv BPS na formaci dělícího vřetene u MII oocytů

Cílem bylo zhodnotit vliv BPS na tvorbu a uspořádání metafázního vřetene MII oocytů ošetřených BPS *in vivo*, zrajících v podmínkách *in vitro*. Bylo zjištěno, že uspořádání chromatinu u MII oocytů, zastavených v 2. meiotickém bloku po kultivaci *in vitro*, nevykazovalo žádné významné rozdíly oproti kontrolní skupině, tj. nebyly detekovány žádné malformace metafázní destičky.

Oproti tomu však byly u těchto oocytů detekovány nekonjugované tubuly v celé délce a pólech vřetene a ve skupině BPS2 byla pozorována dvojitá metafázní vřetena, která však nevykazovala žádné známky malformace (obr. 7A, B). Lze tedy předpokládat, že námi použité dávky BPS primárně vyvolávají poškození vřetene, spíše než nesourodost chromatinu (obr. 7B). Navíc, nebyly pozorovány žádné defekty vřetene po ošetření 0,1 ng BPS x g tělesné váhy^{-1} den^{-1} (BPS2) (obr. 7B), což naznačuje, že tato nízká dávka BPS vykazuje na vřeteni závislé specifické způsoby působení.

Obrázek 7



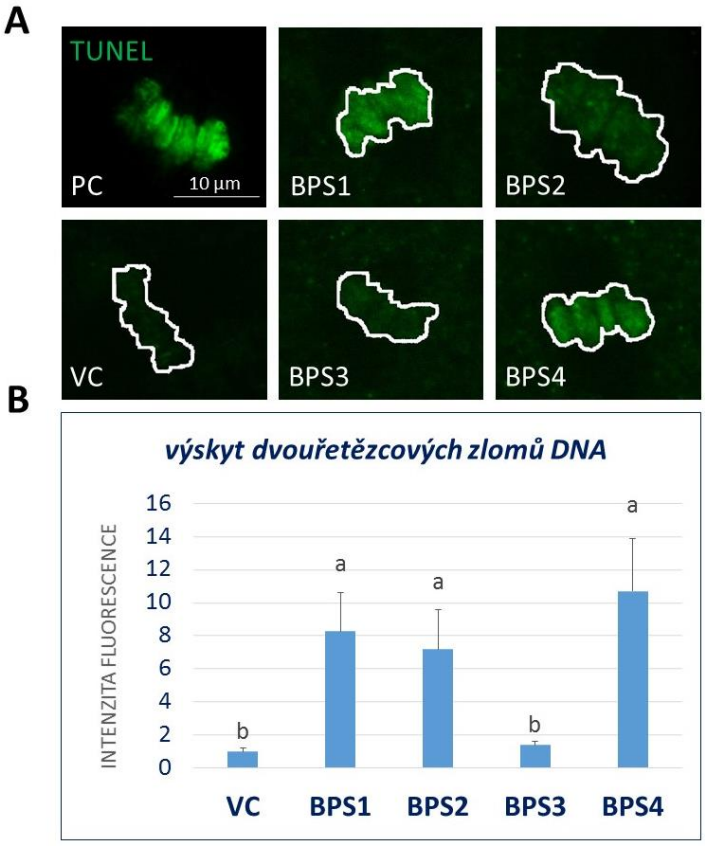
Meiotické vřeteno a zarovnání chromozomů u oocytů zrajících in vitro za přítomnosti BPS. (A) Reprezentativní snímky chromatinu a meiotického vřetene zralých MII oocytů. Samice myší byly vystaveny různým dávkám BPS (BPS1 – BPS4) nebo kontrolnímu roztoku (GV). Chybné zarovnání chromozomů a malformace vřetene byly hodnoceny na základě barvení DNA, respektive α – tubulinu. Šipky označují jednotlivé odchylky chromatinu a trojúhelníky ukazují nekonjugované tubuly v délkách a na pólech vřetene. (B) Chromatin a malformace vřetene byly identifikovány ve čtyřech nezávislých experimentech. Změny jsou

vyjádřeny jako kumulativní podíl normálních a abnormálních oocytů, včetně oocytů s normálním vs. abnormálním chromatinem a vřeteny. Chybové úsečky ukazují 95 % konfidenční intervaly pro proporce populace. Různé horní indexy označují statistickou signifikanci při P <0,05 a P <0,01, v každém experimentu bylo sledováno minimálně 45 oocytů na skupinu.

<u>Vliv BPS na integritu DNA u MII oocytů</u>

Cílem bylo zhodnotit integritu DNA MII oocytů ošetřených BPS *in vivo*, zrajících *in vitro*, za použití metody TUNEL k analýze. Tato analýza prokázala statisticky významné zvýšení výskytu dvouřetězcových zlomů DNA ve třech skupinách exponovaných BPS (BPS1, BPS2, BPS4) oproti kontrolní skupině, což podporuje myšlenku škodlivého účinku BPS (obr. 8A). Po ošetření 10 ng BPS x g tělesné váhy^{-1} den^{-1} (BPS3) (obr. 8B) nebyl zjištěn významný účinek na integritu DNA, což naznačuje, že tato nízká dávka BPS vykazuje specifické způsoby působení.

Obrázek 8

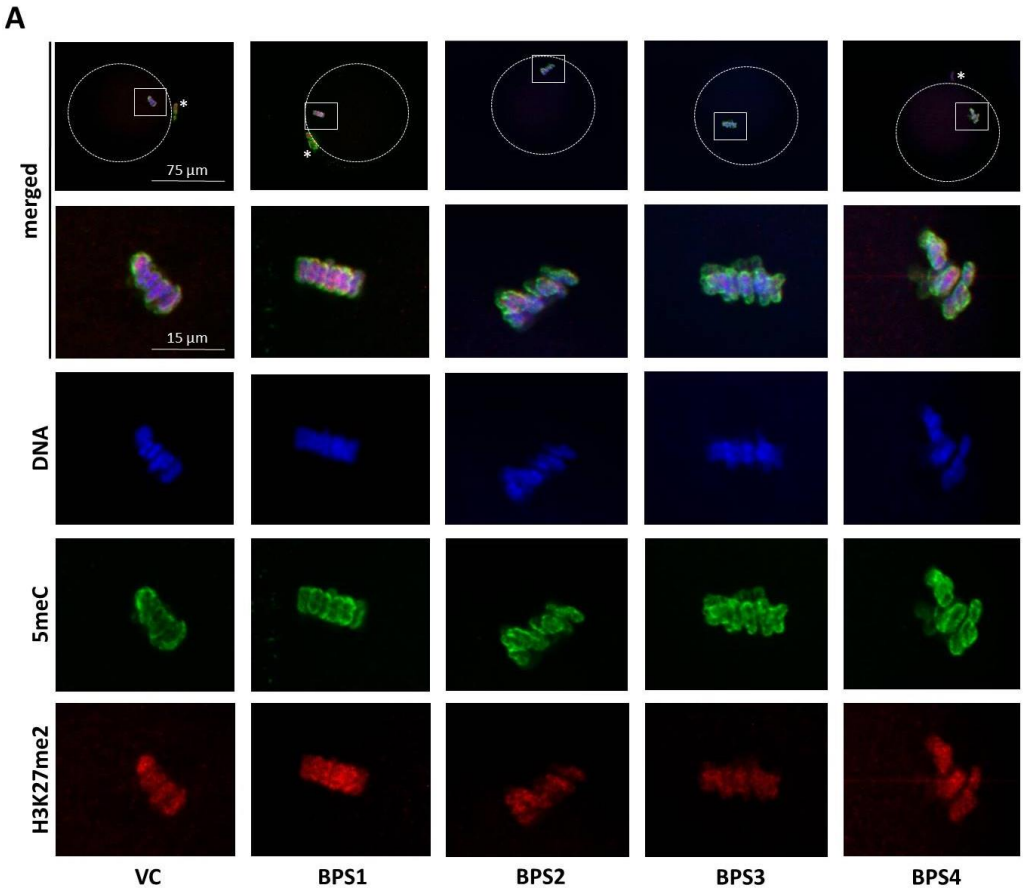


Integrita DNA při zrání oocytů vystavených BPS. (A) Integrita DNA byla hodnocena pomocí testu TUNEL. Obrázky představují integrovanou hustotu vyhodnocenou pomocí programu ImageJ. Samice myší byly vystaveny různým dávkám BPS (BPS1 – BPS4) nebo kontrolnímu roztoku (VC). PC: pozitivní kontrola. (B) Data jsou vyjádřena jako průměr ± SEM ze *čtyř nezávislých experimentů, v každém experimentu bylo sledováno minimálně 15 oocytů na skupinu. Různé horní indexy označují statistický význam při P <0,05 a P <0,01.*

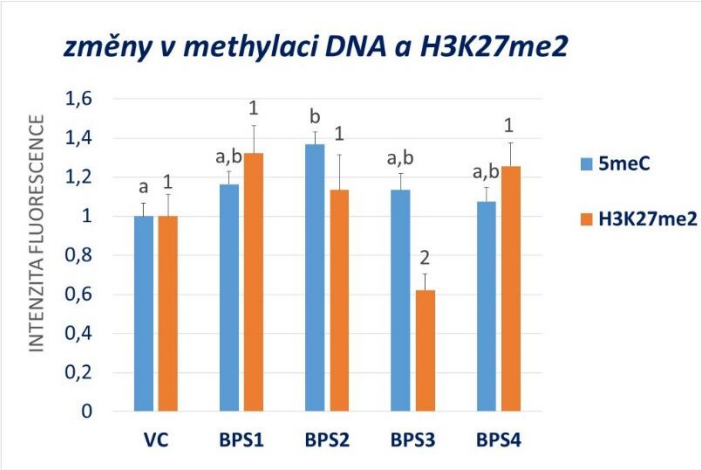
Vliv BPS na změnu v methylaci DNA a histonu H3K27 u MII oocytů

Cílem bylo zhodnotit vliv *in vivo* expozice BPS na epigenetické markery heterochromatinu po zrání oocytů *in vitro*. Imunologické barvení 5meC a H3K27me2 metafázních chromozomů MII oocytů (obr. 9A) odhalilo významně zvýšenou hladinu (P <0,05) 5mC ve skupině BPS2. Hladiny H3K27me2 se však významně nelišily od kontrolních oocytů (obr. 9B). Navíc, hladiny 5meC se významně zvýšily v oocytech ošetřených 0,1 ng BPS x g tělesné váhy^{-1} den^{-1} (BPS2), ačkoli u GV oocytů nebyl pozorován žádný efekt.

Obrázek 9



B



Celogenomové epigenetické markery ve zralých MII oocytech. (A) Reprezentativní snímky 5meC a H3K27me2 ve zralých MII oocytech. Přerušovaný kruh ukazuje hranici oocytů. Rámeček představuje zvýrazněnou oblast metafázního chromatinu. Vydělené pólové tělísko, které označuje zralý oocyt, je označeno hvězdičkou. (B) Integrovaná hustota signálu 5mC a H3K27me2 byla porovnána s hustotou kontrolní skupiny. Data jsou vyjádřena jako průměr ± SEM ze čtyř nezávislých experimentů, v každém experimentu bylo sledováno minimálně 20 oocytů na skupinu. Různé horní indexy označují statistický význam při P <0,05 nebo P <0,001.

7. Diskuse

BPS je v současné době využíván v mnoha běžných spotřebních výrobcích, zejména v produktech označovaných BPA free, kde často slouží jako náhrada BPA, který je prokázaným ED a jeho použití je v EU i USA právně regulováno (EFSA, 2011; FDA, 2012). Vzhledem k tomu, že nedávné biomonitoringové studie zaznamenaly srovnatelný výskyt BPS s výskytem BPA jak v prostředí, tak v některých tělních tekutinách člověka (shrnuto ve Wu *et al*., 2018; Karrer *et al*., 2020), neustále vzrůstá potřeba zabývat se výzkumem biologického účinku BPS k posouzení rizika expozice BPS na lidské zdraví (Rochester and Bolden, 2015). Dosud byly výzkumy zabývající se negativními účinky BPS v souvislosti s lidskou reprodukcí zaměřené zejména na chronickou expozici, tedy vliv dlouhodobé přítomnosti BPS v organismu na reprodukci člověka. Vzhledem ke každodenní opakované expozici člověka nízkým hladinám BPS, který je rychle metabolizován a jeho glukuronidy jsou vylučovány močí (Skledar *et al*., 2016), je ale nezbytné analyzovat také dopady krátkodobé – akutní, zejména s vědomím, že akutní a chronické dopady endokrinních disruptorů na reprodukční zdraví člověka se mohou významně lišit (Da Silva *et al*., 2019), jak již bylo prokázáno ve studiích zabývajících se BPA (Li *et al*., 2016; Berger *et al*., 2008). Pro potřeby této studie jsme zvolili dva experimentální modely – myší a prasečí. Myší experimentální model je díky svému krátkému generačnímu cyklu vhodným modelem pro studium transgeneračních efektů. Jelikož bylo prokázáno, že v citlivém fetálním modelu *in vitro* jsou lidské gonadální buňky 10 až 100krát citlivější na BPA, BPS a bisfenol F (BPF), než buňky hlodavců (Eladak *et al*., 2015), bylo jako druhý experimentální model zvoleno prase, jež je díky analogiím s lidským organismem z hlediska anatomie, genetiky, fyziologie, ale i délky a průběhu meiotického zrání vhodným modelem ke studiu působení xenobiotik na oogenezi (Swindle *et al*., 2012).

Vzhledem k tomu, že v současné době již existují studie prokazující přítomnost BPS v séru a folikulární tekutině matek (Dimitriadis *et al*., 2017; Li *et al*., 2020), využili jsme v naší studii pro simulaci podmínek zrání oocytů, během kterého jsou známy přibližné hladiny koncentrací BPS ve folikulární tekutině samice, prasečí experimentální model *in vitro*. Naopak pro simulaci průběhu meiotického zrání bez přítomnosti BPS, před jehož zahájením došlo k akutní expozici organismu matky přesně definovaným nízkým dávkám BPS, byl využit myší experimentální model. Zvolené testované dávky BPS byly nižší, než koncentrace BPS pozorované v lidské moči a krevním séru (Thayer *et al*., 2016). Ačkoli již velmi nízké dávky

BPS mají známá rizika (Eladak *et al*., 2015), tolerovatelná denní dávka (tolerable daily intake; TDI) pro BPS dosud nebyla stanovena. BPS vyskytující se v prostředí člověka si zalouží zvýšenou pozornost, zejména pokud jde o lidské reprodukční zdraví a jeho akutní / chronické účinky.

Pro analýzu potenciálního efektu expozice BPS na meiotickou progresi prasečích oocytů bylo nejdříve hodnoceno jaderné zrání oocytů v průběhu rozpadu jaderné membrány (GVBD), která tvoří přirozenou bariéru mezi jadernými a cytoplazmatickými složkami oocytu a jak bylo již prokázáno u myší, průběh GVBD může být zpožděn, nebo inhibován působením BPA (Chao *et al*., 2012), BPAF (Ding *et al*., 2017), nebo BPB (Zhang *et al*., 2020). Přesto, že v našich experimentech nedošlo k žádným statisticky významným změnám v progresi jaderného zrání během GVBD, byl pozorován trend počátečního zrychlení GVBD, které bylo následováno postupným zpomalením průběhu GVBD, což by mohlo souviset se změnou aktivity MAPK, která se na GVBD významně podílí a může být ovlivněna působením bisfenolů, jak již bylo prokázáno u BPA (Wang *et al*., 2016).

Dále bylo hodnoceno jaderné zrání v průběhu MI a MII, jež tvoří další významná meiotická stádia, nezbytná pro úspěšné zrání, oplození a následný embryonální vývoj (Aebi *et al*., 1986). Oocyty, které byly ošetřeny BPS, vykazovaly signifikantní, na dávce závislé snížení schopnosti dosáhnout MI a MII po 24 a 48 hodinách *in vitro* kultivace. Po 24 hodinách kultivace dosáhly oocyty stádia MI se sníženou úspěšností, zatímco všechny oocyty dosáhly stádia MI po 48 hodinách zrání, ale část nepokračovala v meióze až do MII. Období kolem MI se tak zdá být vzhledem k pozorovaným účinkům BPS na zrání oocytů prasat kritické, čemuž odpovídají i naše předešlá pozorování (Žalmanová *et al*., 2017). BPS způsobuje nejen zpomalení zrání prasečích oocytů *in vitro*, podobné tomu, které bylo pozorováno ve vyšších koncentracích za přítomnosti bisfenolu AF (Nakano *et al*., 2016), nebo BPA během zrání prasečích (Wang *et al*., 2016) a myších (Can *et al*., 2005) oocytů, ale BPS také blokuje průběh zrání ve významné části oocytů, jak již bylo pozorováno u lidských (Machtinger *et al*., 2013), myších (Lenie *et al*., 2008), prasečích (Wang *et al*., 2016) a bovinních (Ferris *et al*., 2015) oocytů za přítomnosti BPA. Podobné účinky BPS ve vyšších koncentracích, než byly použity v našich experimentech, byly již pozorovány při zrání bovinních a ovčích oocytů (Campen *et al*., 2018; Desmarchais *et al*., 2020).

Citlivost oocytů vůči BPS během období kolem MI souvisí s vlivem na tvorbu meiotického vřetene. BPS narušuje jeho tvorbu v prasečích oocytech a způsobuje nepravidelnosti v uspořádání tubulinových vláken, což má negativní vliv na organizaci chromozomů a progresi buněčného cyklu (Žalmanová *et al*., 2017). Tyto účinky lze připsat estrogenní aktivitě BPS, konkrétně napodobení účinku estradiolu, který ovlivňuje regulaci zrání oocytů savců *in vitro* (Beker *et al*., 2002; Beker – van Woudenberg *et al*., 2004) a zvýšené koncentrace mají za následek defekty meiotického vřeténka (Beker – van Woudenberg *et al*., 2004). Podobné účinky, vyvolávající chybnou organizaci chromozomů *in vivo* (Nevoral *et al*., 2018) a *in vitro* (Can *et al*., 2005; Eichenlaub – Ritter *et al*., 2008), již byly pozorovány během zrání myších a bovinních (Nevoral *et al*., 2018; Campen *et al*., 2018) oocytů v přítomnosti BPS; abnormality meiotického vřetene byly zjevně výsledkem selhání SAC (spindle assembly checkpoint).

Ačkoliv negativní účinky BPS na jaderné zrání v průběhu GVBD nebyly statisticky významné, pozorovaný trend napovídá, že i tato počáteční fáze zrání by mohla být citlivá na působení BPS. Období GVBD je iniciováno postupným rozpadem proteinové sítě vnitřní strany jaderné membrány a její interakcí s chromatinem. Tato síť se podílí na přeuspořádání a kondenzaci chromatinu v průběhu GVBD (Chakarova *et al*., 2017), proto je pravděpodobné, že zpomalení, nebo zastavení jaderného zrání prasečích oocytů může být spojeno i s rozpadem proteinové sítě vnitřní strany jaderné membrány. Z tohoto důvodu byly naše další experimenty zaměřeny právě na jadernou membránu a na proteinovou síť vnitřní strany membrány – jadernou laminu, která iniciuje GVBD a fosforylace proteinů jaderné laminy může být ovlivněna působením BPS.

Na základě naší hypotézy byl testován vliv BPS na lokalizaci laminových proteinů (lamin A / C) v průběhu všech tří vybraných stádií, zvolených v předchozích experimentech – GVBD, MI a MII. U všech experimentálních skupin došlo k významnému zpoždění rozpadu jaderné membrány oproti kontrolním oocytům. Zatímco u kontrolních oocytů došlo k počátečnímu kolapsu membrány již ve stádiu GV2, oocyty ošetřené BPS zahájily viditelný rozpad membrány až ve stádiu GV4. Jednou z potenciálních příčin tohoto efektu může být rozdílná fosforylace laminových proteinů po ošetření oocytů BPS, způsobená ovlivněním aktivity MAPK, která patří mezi významné faktrory regulující zrání oocytů a jejíž signální

dráha je zodpovědná za znovuzahájení meiotického zrání, fosforylaci laminových proteinů a organizaci vřeténka v prasečích oocytech (Liang *et al*., 2007; Sun *et al*., 2016). Změna aktivity MAPK může vést ke zpoždění a / nebo úplnému selhání GVBD, chybnému zarovnání metafázních chromozomů do ekvatoriální roviny, vydělení prvního pólového tělíska a může vést až k úplnému zastavení meiotického zrání, nebo tvorbě aneuploidií po oplození, jak již bylo prokázáno u prasečích (Wang *et al*., 2016) a myších (Can *et al*., 2005) oocytů vlivem působení BPA.

Navíc, fragmenty jaderné membrány byly v našich experimentech u oocytů ošetřených BPS pozorovány i ve stádiu MI, ve kterém by vlivem disperzního rozptýlení po cytoplazmě oocytu neměly být lokalizovatelné (Prentice – Biensch *et al*., 2012). Zároveň byl v našich experimentech u ošetřených oocytů pozorován i rozdílný způsob GVBD, doprovázený vchlipováním jaderné membrány do nukleoplazmatického prostoru. Tento efekt může být způsoben opožděným oddělením jaderné laminy od chromatinu, což může souviset s perzistencí fragmentů jaderné membrány ve stádiu MI a opožděním až zástavou jaderného zrání. Podobný efekt byl pozorován ve studii zabývající se vlivem exprese nefosforylovatelného laminu na průběh mitotického dělení u zygot (Velez – Aguilera *et al*., 2020), či studii sledující vliv post – translačních modifikací laminu A / C na průběh meiotického zrání u *Caenorhabditis elegans* (Link *et al*., 2018). Uvedená pozorování podporují domněnku vlivu BPS na fosforylaci laminů vlivem aktivity MAPK a naše další experimenty budou zaměřeny právě na tuto problematiku.

Pro simulaci podmínek v těle matky, akutně exponované nízkým dávkám BPS z prostředí, jsme v následující části našich experimentů zvolili myší samice akutně vystavené BPS *in vivo* a následně jejich oocyty, podstupující meiotické zrání *in vitro* bez přítomnosti BPS. Heterogenita v lidské populaci byla simulována pomocí hormonálně nestimulovaného outbredního myšího kmene. Pro zkoumání možného dopadu akutní expozice BPS na meiotickou progresi oocytů byla nejprve vyhodnocena výtěžnost GV oocytů, meiotická způsobilost oocytů (SN vs. NSN oocyty), opětovné zahájení meiotického zrání a rychlost zrání. Poté, co v těchto parametrech nebyly při akutní expozici zaznamenány žádné významné rozdíly, byly následně hodnoceny molekulární markery meiotického zrání oocytů.

Nejprve bylo testováno meiotické vřeteno, které zprostředkovává zarovnání a segregaci chromozomů, jak bylo již popsáno u prasečího experimentálního modelu. Vřeteno také působí jako centrum fyziologicky asymetrické cytokineze oocytů (Grøndahl *et al*., 1988). V našich experimentech bylo ve všech skupinách ošetřených BPS pozorováno zvýšené procento abnormálních oocytů, včetně zvýšeného výskytu malformace vřetene a chybného zarovnání chromozomů. Zejména nepravidelné uspořádání mikrotubulů vřetene bylo nejčastějším pozorovaným fenotypem, což zdůrazňuje účinek BPS podobný estrogenu (Beker – van Woudenberg *et al*., 2004) a zároveň tyto výsledky naznačují, že akutní expozice BPS může způsobovat spíše poškození vřetene než vychýlení chromatinu.

Dále byla hodnocena integrita chromatinu kvantifikací dvouřetězcových zlomů DNA. Negativní účinek BPS na integritu DNA, indikovaný zvýšeným signálem TUNEL, byl detekován ve skupinách BPS1, BPS2 a BPS4. Toto zjištění potvrzuje nemonotónní reakci na expozici BPS, která byla dříve popsána jako charakteristická pro efekty ED (Vandenberg *et al*., 2012). Výsledky našich experimentů akutního působení BPS na myším experimentálním modelu se sníženou hormonální stimulací tedy podporují hypotézu negativního účinku BPS.

Kromě poškození cytoskeletu mají endokrinní disruptory také dobře definovaný epigenetický účinek. V našich experimentech bylo zjištěno, že jak methylace DNA, tak methylace histonu (tj. 5meC a H3K27me2), jako markery genomické represe, jsou náchylné k narušení po akutní expozici BPS. V nezralých GV oocytech byla po ošetření BPS3 zvýšena methylace H3K27. Naproti tomu hladiny 5meC vzrostly ve zralých MII oocytech ve skupině BPS2. Naše pozorování tedy podporují dříve pozorovaný účinek BPA na methylaci histonu v oocytech (Trapphoff *et al*., 2013; Wang *et al*., 2016) a změny H3K27me2 vyvolané účinkem BPS (Nevoral *et al*., 2018). Epigenetický účinek se však lišil podle způsobu expozice (akutní vs. chronická). Hladina H3K27me2 se zvýšila po chronické expozici BPS (Nevoral *et al*., 2018), zatímco u současných experimentů s akutní expozicí nebyl pozorován žádný účinek. Tato nesrovnalost zdůrazňuje možné různé cíle akutní vs. chronické expozice, zejména preantrální / antrální folikuly a dřívější stadia (tj. primordiální a / nebo primární folikuly). Dohromady tato zjištění naznačují, že BPS moduluje epigeneticky řízenou genovou expresi, podobně jako BPA (Verbanck *et al*., 2017). Kromě toho může působení BPS vyvolat změny v epigenomickém programování, které může přetrvávat po celý život, a tím měnit genovou

expresi a prostřednictvím transgeneračního přenosu ovlivňovat i následující generace (Walker, 2016).

Naše výsledky souhrnně naznačují, že je nutné identifikovat molekulární cíle a interakce BPS v gametách a časných embryích s cílem chránit lidské reprodukční zdraví. Pro úplné objasnění jsou nutné další experimenty, zaměřené na oplodnění a časný embryonální vývoj oocytů ovlivněných BPS, s přihlédnutím ke kvalitativním změnám mezi oocyty zrajícími *in vitro* a *in vivo*, hormonální stimulaci (pokud jsou využity technologie asistované reprodukce) a vývojové úspěšnosti embryí po přirozeném oplodnění nebo oplodnění *in vitro*.

8. Závěr

Meiotické zrání oocytů je klíčovým procesem pro oblast reprodukčních biotechnologií, jako je *in vitro* oplození (IVF) nebo přenos jader somatických buněk (SCNT). Detailní studium meiotického zrání oocytů a testování potenciálních látek, které by mohly bezchybný průběh zrání narušit, je nezbytné pro vyšší efektivitu získávání *in vitro* dozrálých oocytů a tedy další pokrok reprodukčních biotechnologií s aplikacemi v humánní medicíně. Cílem práce bylo ověřit hypotézu, podle které široce rozšířený endokrinní disruptor bisfenol S (BPS) negativně ovlivňuje průběh meiotického zrání prasečích a myších oocytů *in vitro*.

Na základě experimentů bylo zjištěno, že bisfenol S významně narušuje průběh meiotického zrání prasečích oocytů *in vitro*, a to jak v průběhu rozpadu jaderné membrány (GVBD), tak během metafáze I (MI) a metafáze II (MII). Expozice BPS během meiotického zrání sice neovlivnila schopnost oocytů dosáhnout jednotlivých fází jaderného zrání během GVBD, významně však ovlivnila rozpad a způsob rozpadu jaderné membrány a také schopnost oocytů dosáhnout metafáze I a metafáze II. Lze předpokládat, že opoždění rozpadu jaderné membrány je pravděpodobně spojeno s její zpožděnou depolymerací, která vyvolává perzistenci fragmentů membrány ve stádiu MI, ve kterém je za fyziologických podmínek jaderná membrána v oocytu již nepřítomna.

Další experimenty byly zaměřeny na vliv akutní expozice BPS *in vivo* na následný průběh meiotického zrání myších oocytů *in vitro*. Bylo zjištěno, že akutní expozice samic před zahájením meiotického zrání neměla vliv na výtěžnost oocytů ani meiotickou způsobilost. Nebyl pozorován statistický významný efekt ani na úspěšnost zrání oocytů, na rozdíl od prasečích oocytů, kde byl významný efekt BPS na úspěšnost zrání prokázán. Je pravděpodobné, že rozdílný efekt BPS na úspěšnost zrání u prasečích a myších oocytů je dán rozdílným způsobem expozice, zvolenou dávkou a druhově specifickými nároky na zrání.

Dále byly u myších oocytů sledovány celogenomové markery stability heterochromatinu, a to jak ve stádiu GV, tak u zralých MII oocytů. Bylo zjištěno, že jak methylace DNA, tak methylace histonu (5meC, H3K27me2), jako markery genomické represe, jsou náchylné k narušení po akutní expozici BPS. Zatímco v nezralých GV oocytech došlo k významnému zvýšení methylace H3K27 (BPS3), hladiny 5meC v MII oocytech významně vzrostly ve skupině BPS2. Tato zjištění naznačují, že BPS moduluje epigeneticky řízenou genovou expresi, podobně jako BPA.

Také experimenty zaměřené na defekty chromatinu a formování meiotického vřetene prokázaly negativní vliv BPS na meiotické zrání myších oocytů. Byla pozorována tvorba nekonjugovaných tubulů (BPS1, BPS3, BPS4) a tvorba dvojitého vřetene (BPS2), zatímco nebyly prokázány žádné malformace metafázní destičky, bylo však pozorováno významné zvýšení výskytu dvouřetězcových zlomů DNA (BPS1, BPS2, BPS4).

Naše výsledky potvrzují účinky BPS jako endokrinního disruptoru, přesto je zapotřebí dalších výzkumů, které by detailněji objasnily mechanismus účinku BPS. Z našich výsledků vyplynulo, že expozice velmi nízkým dávkám BPS významně ovlivňuje kvalitu oocytů během meiotického zrání, zejména proces GVBD, tvorbu vřetene, integritu DNA a epigenetické modifikace nukleozómů. Naše pozorování proto naznačují, že BPS není vhodnou náhradou za BPA, produkce BPS by měla být výrazně omezena nebo zakázána a kritéria pro zvolení vhodné alternativy by měla být nastavena přísněji.

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10. Samostatné přílohy

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Acute low-dose bisphenol S exposure affects mouse oocyte quality

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ABSTRACT

Bisphenol S (BPS) is widely used to replace the known endocrine disruptor BPA in various products. We evaluated the effect of acute *in vivo* BPS exposure on oocyte quality, simulating the oral route of exposure via oral gavage. Eight-week-old ICR female mice (N = 15 per experimental group) were exposed to vehicle or BPS1–BPS4 (0.001, 0.1, 10, and 100 ng BPS x g bw^{-1} day^{-1}, respectively) for seven days. Oocytes were isolated and matured *in vitro*. We observed that BPS exposure increased aberrant spindle formation in mature oocytes and induced DNA damage. Moreover, BPS3 significantly increased the chromatin repressive marks 5-methyl cytosine (5meC) and H3K27me2 in immature oocytes. In the BPS2 group, the increase in 5meC occurred during oocyte maturation. Transcriptome analysis revealed differential expression of early embryonic development transcripts in BPS2-exposed oocytes. These findings indicate that the biological effect of BPS is non-monotonic, affecting oocyte quality even at concentrations that are orders of magnitude below those measured in humans.

1. Introduction

Bisphenols are widely used in day-to-day consumer products including paper, cans, and baby bottles [1,2]. The most widely used bisphenol, bisphenol A (BPA), constitutes an endocrine disruptor with numerous deleterious effects on public health [3]. Further, very low (e.g., subtoxic) doses negatively affect health with a nonlinear effect [4,5]. These findings have led to BPA elimination based on tolerable daily intake (TDI) thresholds, including 25 ng x g body weight $(bw)^{-1}$ day^{-1} as determined by the United States Food and Drug Administration in 2014 [6] and more strictly as 4 ng x g bw^{-1} day^{-1} by the European Food Safety Administration in 2015 [7], following a complete ban on BPA in children's items and additional 'BPA-free' products by the latter agency in 2013 [8]. However, the resulting products are not truly bisphenol-free.

In particular, Bisphenol S (BPS) has become the most widely used replacement for BPA because it is more chemically stable and economical to use [9]. Although released into the environment in lower amounts than BPA [10], BPS is often detected in the environment, including in the air, water, food, and/or house dust [11]. Accordingly, increasing human BPS exposure has been confirmed [12]. Analogous to well-known BPA exposure routes, BPS enters the human body via ingestion, inhalation, or dermal contact [13]. Following entry into the body, BPS is rapidly metabolised and gradually excreted. However, despite such intensive BPS clearance, repeated acute human exposure is probable owing to the high stability and prevalence of BPS in the environment [14].

The body responds to endocrine disruptors in a nonlinear manner,

Abbreviations: 5meC, 5-methyl cytosine; BPA, bisphenol A; BPS, bisphenol S; bw, body weight; DAPI, 4′,6-diamidino-2-phenylindole; GV, germinal vesicle; GVBD, germinal vesicle breakdown; IBMX, isobutyl-methylxanthine; MII, metaphase II; NSN, non-surrounded nucleolus; PBS, phosphate buffered saline; qRT-PCR, quantitative reverse transcription-polymerase chain reaction; SN, surrounded nucleolus; TDI, tolerable daily intake; TUNEL, Terminal deoxynucleotidyl transferase dUTP nick end labelling

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with paradoxically stronger effects at lower doses [5]. Recent BPS exposure in the general population ranges from 0.01 to 10 ng/mL in several body fluid types including the blood, urine, breast milk, and follicular fluid [15–17]. The highest levels occur among individuals with occupational exposure [18], with an estimated daily intake of 0.06–1.7 ng × g bw^{-1} [11,19]. The biomonitoring data indicate a possible risk of such low doses for human health if the negative effects of BPS replicate those of BPA.

Notably, the reproductive system is a unique indicator of detrimental impact consequent to bisphenols and other hormonal disruptors in the environment owing to their stimulation of estrogenic signalling [20], alteration of post-translational modifications of functional proteins [21], and epigenetic shift in germ cells [22], which opens the possibility of transmitting the effect into subsequent generation(s) [23,24]. The female reproductive system is particularly extremely sensitive to environmental stress because the oocyte pool is not renewed. Furthermore, multiple features in this system may be adversely affected, thus impacting fertilisation and embryonic development [25]. Specifically, the two physiological checkpoints where oocyte meiosis is arrested represent critical exposure windows of oocyte susceptibility. Chromatin assembly initially occurs in germinal vesicles (GVs) in immature oocytes and is arrested at the meiotic prophase checkpoint [26]. Mouse prophase oocytes isolated from antral follicles in the first critical window may possess two chromatin configuration types. The first is the non-surrounded nucleolus (NSN) configuration, with more dispersed chromatin and high transcriptional activity characteristic of growing oocytes. The second is the transcriptionally inactive surrounded nucleolus (SN) configuration, which is attained upon oocyte growth and exhibits more condensed chromatin, with a significant fraction concentrated around the nucleolus [27]. Thus, fully grown oocytes isolated from antral follicles represent a non-homogenous population, in which oocytes have either NSN-type or SN-type chromatin conformation [28]. On such a background, epigenetic GV marks constitute a precise tool to evaluate oocyte health because oocyte chromatin uniqueness results from transcriptional silencing and many endocrine disruptors have an epigenetic mode of action [29,30]. Moreover, oocyte maturation, fertilisation, and embryonic development are fully dependent on the oocyte cytoplasmic mRNA pool and organelles. Contrary to the *status quo* description of immature oocytes, chromatin dynamics during oocyte maturation is essential. The chromatin changes necessary for successful fertilisation and embryonic development facilitate 1) restarting meiosis after the first checkpoint, manifested by GV breakdown (GVBD); 2) proper chromosome segregation and reduction to haploid order, leading to polar body extrusion; 3) chromosome alignment in the metaphase II (MII) plate; and 4) establishment of the second meiotic checkpoint and chromatin integrity as an outcome of DNA damage [31,32].

In mammalian oocytes, very low BPS doses cause various deleterious impacts in pig oocytes matured *in vitro* [33] and in mouse oocytes *in vivo* [34]. In these studies, BPS exposure causes meiotic spindle formation failure, improper chromosome alignment, and alters oocyte oestrogen receptor expression and distribution [33]. In our previous study, we demonstrated that long-term exposure to very low BPS doses causes decreased antral follicle size and number in female mice [34]. In addition to causing cytoskeletal changes in the oocyte spindle, BPS exposure results in inadequate genome-wide epigenetic changes [34]. However, the cytoskeletal and epigenetic disturbances in oocytes consequent to doses present in human populations remains unclear.

Therefore, the aim of this study was to assess both cytoskeletal and chromatin changes in oocytes following *in vivo* exposure, using appropriate markers. Moreover, mRNA analysis was performed to profile transcriptome-wide changes and predict the impact of BPS-affected oocytes on subsequent fate. Accordingly, we simulated acute oral exposure with wide-range subtoxic BPS doses *in vivo*, causing immature GV oocytes to arrest at the first meiotic checkpoint. To track the hormonally disruptive effects of BPS, we assessed the quality of immature oocytes, spindle assembly, chromatin integrity, and epigenetic modifications in mice without hormonal stimulation.

2. Materials and methods

2.1. Reagents

All chemicals were purchased from Sigma-Aldrich (USA) unless stated otherwise.

2.2. Animal use and housing

All animal procedures were conducted in accordance with Act No. 246/1992 Coll. on the Protection of Animals against Cruelty under the supervision of the Animal Welfare Advisory Committee at the Ministry of Education, Youth and Sports of the Czech Republic, approval ID MSMT-11925/2016-3. Six- to seven-week-old female ICR mice were purchased from Velaz Ltd. (Czech Republic), housed in intact polysulphonate cages, and maintained in a facility with a 12 h light/dark cycle, a temperature of 21 ± 1 °C, and a relative humidity of 60 %. A phyto-oestrogen-free diet (1814 P; Altromin, Germany) and ultrapure water (in glass bottles, changed twice per week) were provided *ad libitum*. Animals were allowed to acclimate for at least one week prior to initiation of treatment.

2.3. BPS exposure experiments

Animals (N = 75) were randomly separated into five experimental groups of 15 animals per group treated with one of four different BPS doses (0.001, 0.1, 10, and 100 ng BPS x g bw^{-1} day^{-1}, hereafter termed BPS1, BPS2, BPS3, and BPS4, respectively), and vehicle (see below). Experiments were performed in five independent replicates. BPS was dissolved in 50 µl 50 % glycerol containing 0.1 % dimethylsulphoxide and administered daily for seven days by oral gavage. After the exposure period, mice were euthanised by cervical dislocation and their ovaries were collected for further experiments.

2.4. Oocyte collection and in vitro maturation

Ovarian follicles were punctured using 27 gauge needles. Immature oocytes in the GV stage were collected and manipulated in M2 medium supplemented with 100 µM isobutyl-methylxanthine (IBMX), a specific endogenous phosphodiesterase inhibitor, to maintain intact GV oocytes [35]. Fully grown immature oocytes with intact GVs were placed in M16 culture medium with IBMX and allowed to recover their oocyte pool of proteins for at least 1 h at 37 °C and 5 % CO_2. Thereafter, oocytes were fixed in 4 % paraformaldehyde in phosphate buffered saline (PBS), supplemented with 0.1 % polyvinyl-alcohol, for 30 min at room temperature (22 °C), and stored at 4 °C until further usage. Alternatively, recovered GV oocytes were cultured in IBMX-free M16 culture medium for 16 h at 37 °C and 5 % CO_2 to obtain matured MII oocytes. Matured oocytes with extruded polar bodies were fixed and stored as described above.

2.5. Terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) assay

Fixed MII oocytes were permeabilised in 0.1 % Triton X-100 in PBS containing 0.05 % NaN_3 for 40 min. The oocytes were treated with fluorescein-conjugated dUTP and terminal deoxyribonucleotidyl transferase enzyme (In Situ Cell Death Detection Kit, cat. No. 11684795910, Roche, Germany) for 1 h in the dark at 37 °C. The positive control was prepared using a DNase I kit (AMP-D1, Sigma-Aldrich). Finally, the oocytes were mounted onto slides with Vectashield medium plus 4′,6-diamidino-2-phenylindole (DAPI; Vector Laboratories Inc., USA). Signal intensity was measured using ImageJ software (National Institutes of

Health, USA).

2.6. Immunocytochemistry and image analysis

Fixed oocytes were permeabilised in PBS containing 0.04 % Triton X-100 and 0.3 % Tween-20 for 15 min. Oocyte heterochromatin marks were evaluated including 5′-methyl cytosine (5meC) and dimethylation of histone H3 on lysine K27 (H3K27me2). 5meC-H3K27me2 co-staining was performed using HCl and trypsin as previously described [36]. Then, oocytes were blocked in 1 % bovine serum albumin in PBS with Tween 20 for 15 min and incubated with anti-α-tubulin (1:200, Sigma-Aldrich) or a cocktail of anti-5meC (1:200, Sigma-Aldrich) and anti-H3K27me2 (1:200, Abcam, UK) antibodies. After washing, the oocytes were incubated with a cocktail of anti-mouse and anti-rabbit AlexaFluor 488 and 647 (1:200) antibodies, respectively. Phalloidin (1:200; Thermo Fisher Scientific, USA) was added to washes and used for β-actin visualisation. Stained oocytes were mounted onto slides in Vectashield medium with DAPI. Signal intensity was measured using ImageJ software. Chromatin configuration around the nucleolus (SN, NSN [37]) and extrusion of the polar body were also evaluated. Images were acquired using an Olympus IX83 spinning disc confocal microscope (Olympus, Germany) and VisiView software (Visitron Systems GmbH, Germany).

2.7. RNA isolation

For each sample, 50 oocytes were collected in TRIzol reagent (Invitrogen, USA) and homogenised using a TissueLyser LT (Qiagen, The Netherlands) for 5 min. Chloroform was used for phase separation, and the aqueous phase was mixed 1:1 with 70 % ethanol. RNA was purified using RNeasy MinElute spin columns (Qiagen) and quantified by quantitative reverse transcription-polymerase chain reaction (qRT-PCR; see section 2.8). Integrity was assessed using a 2100 Bioanalyzer and an RNA 6000 Pico Kit (Agilent, Germany).

2.8. qRT-PCR

Total RNA was reverse transcribed using SuperScript III (Thermo Fisher). Mouse phosphoglycerate kinase (*Pgk1*) cDNA was amplified using the following primers: Pgk1_c140F: 5′ GGTGTTGCCAAAATGTC GCT 3′ and Pgk1_c186R: 5′ AACGGACTTGGCTCCATTGT 3′; 186 bp amplicon size. The amplification reaction was performed in an Applied Biosystems 7900 H T thermal cycler using PowerUp SYBRGreen master mix (Thermo Fisher). *Pgk1* was selected from among three possible housekeeping genes based on stability and high oocyte expression. RNA samples were diluted to equal concentrations based on the relative *Pgk1* quantity (computed using the $2^{-\Delta Ct}$ method).

2.9. Microarrays

Transcriptome expression analysis was performed with four control samples and four BPS-exposed samples using Affymetrix Mouse Gene 2.1 ST Array Strips (USA). Each strip evaluated two controls and two exposed samples. The results were analysed using the Transcriptome Analysis Console (TAC, Affymetrix). The data discussed in this study have been deposited in the NCBI Gene Expression Omnibus [38,39] and are accessible through GEO Series accession number GSE140640 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE140640). We selected quantile normalisation and assessed BPS exposure as the main factor, controlling for scan date (i.e., the strip) as the confounding variable. Statistical differences were tested using two-way analysis of variance (ANOVA), with multiple comparison correction using the Benjamini-Hochberg (false discovery rate) method.

2.10. Statistical analysis

The data were processed using Statistica Cz 12 (StatSoft, Inc., USA). Kruskal–Wallis ANOVA was used for quantitative variables and chi-square tests were used for proportions. Significant differences between individual group pairs were assessed *post hoc* using multiple comparisons of mean ranks, Mann–Whitney U tests with Bonferroni correction, or Fisher's exact tests with Bonferroni correction. Where appropriate, correlations among variables were assessed using Spearman's method. Statistical significance was set at $\alpha = 0.05$, and all reported *P*-values and tests were two-tailed.

3. Results

3.1. BPS effects on oocyte quantity and meiotic ability

We first evaluated the effect of BPS on ovarian capacity and the maturation rate of GV oocytes isolated from BPS-exposed, hormonally-unstimulated female mice. SN and NSN statuses in GV oocytes and the GVBD/maturation rate in mature oocytes were analysed using DAPI-stained oocyte chromatin. We observed that the GV oocyte yield was not affected by BPS treatment (Fig. 1A). Similarly, the SN/NSN ratio did not differ between the control and BPS groups (Fig. 1B). Even the maturation rate and meiotic capability (Fig. 1D) showed no statistically significant differences, suggesting that BPS exposure at the tested levels in oocyte donors has no effect on the general oocyte quantity or quality.

3.2. Effects of BPS exposure on spindle formation in mature MII oocytes

We next assessed metaphase spindle formation of BPS-treated MII oocytes matured *in vitro*. Chromatin features established at the second matured oocyte arrest stage recapitulated both the molecular and cytoskeletal inheritance of immature GV oocytes exposed to BPS *in vivo*; i.e., no significant differences were detected. However, BPS-treated mature oocytes showed unconjugated tubules on the barrel and pole of the spindle and some double metaphase spindles occurred in the BPS2 group (Fig. 2A). Thus, BPS primarily induced spindle damage rather than chromatin misalignment (Fig. 2B–D). Notably, no consistent effect on spindle malformation was observed following 0.1 ng x g bw^{-1} day^{-1} (BPS2) treatment (Fig. 2D), suggesting that low-dose BPS exerts spindle-specific modes of action.

3.3. Effects of BPS exposure on DNA integrity in mature MII oocytes

DNA integrity of BPS-treated MII oocytes matured *in vitro* was also evaluated. TUNEL assays used to analyse DNA double-strand breaks revealed increased abnormal oocytes and elevated abnormal chromosome alignment and/or spindle malformation in all BPS-exposed groups, supporting the deleterious effect of BPS (Fig. 3A). Remarkably, no consistent effect on DNA integrity was detected following 10 ng x g bw^{-1} day^{-1} (BPS3) treatment (Fig. 3B), suggesting that low-dose BPS also exerts DNA-specific modes of action.

3.4. BPS alters H3K27 methylation in immature GV oocytes

Based on the cytoskeletal and chromatin changes in mature oocytes, we investigated epigenetic alterations to GV oocytes following BPS treatment. As genome-wide DNA methylation and histone H3 constitute markers of heterochromatin establishment and chromatin stability, 5meC and H3K27me2 were evaluated on a global level using immunocytochemistry (Fig. 4A). 5meC levels were significantly increased in the BPS3 ($P < 0.05$) and BPS4 ($P < 0.01$) groups compared to the BPS2 5meC level. However, no significant difference was observed compared to control oocytes (Fig. 4B). In comparison to the control, H3K27 dimethylation was increased in BPS3 oocytes (Fig. 4C).

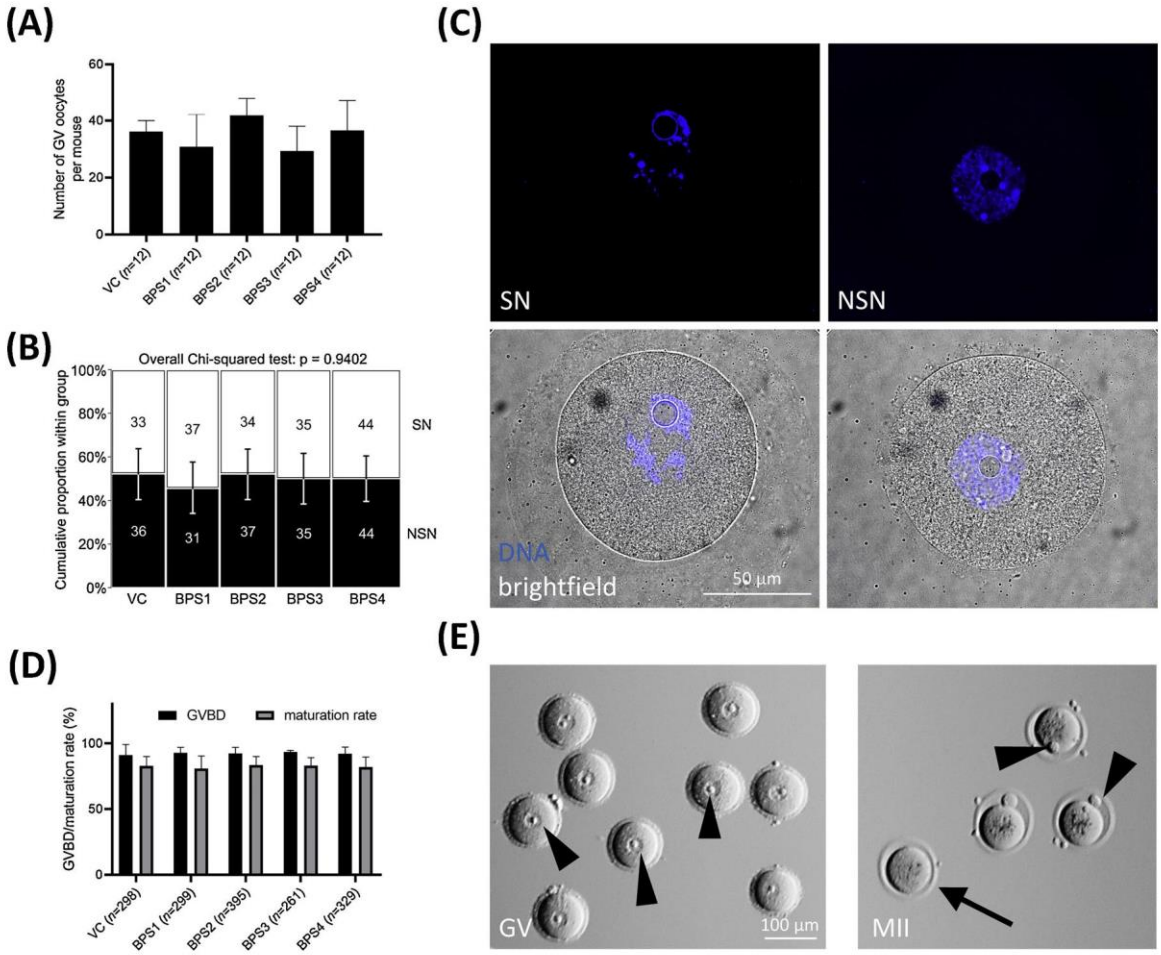

Fig. 1. Quantity and quality of germinal vesicle (GV) oocytes isolated following exposure of female mice to BPS. (A) Oocyte yield based on the number of oocytes isolated from one female. Data are expressed as median, minimum, and maximum lines. Numbers of females from four independent experiments are indicated in brackets. (B) Surrounded (SN) and non-surrounded nucleolus (NSN) proportions from six independent experiments. (C) Representative fluorescent pictures of SN and NSN oocytes in the GV stage. (D) GV breakdown (GVBD) and oocyte maturation rates (median and min–max), indicating GV oocyte capacity to reinitiate meiosis and achieve MII stage, respectively. The numbers of oocytes from five independent experiments are indicated in brackets. (E) Representative pictures of immature GV and matured MII oocytes in transmitted light. The arrow indicates the oocyte having undergone GBVD but not achieving the MII stage. Arrowheads indicate GV and extruded polar body, as markers of immature GV and matured MII oocytes, respectively.

3.5. BPS modifies genome-wide DNA methylation in mature MII oocytes

We next examined heterochromatin marks following *in vitro* oocyte maturation. Immunostaining of 5meC and H3K27me2 in metaphase chromosomes of matured oocytes (Fig. 5A) revealed significantly increased ($P < 0.05$) 5meC in the BPS2 group (Fig. 5B). However, H3K27me2 levels were not significantly different compared to those of controls (Fig. 5C). Notably, 5meC levels increased in BPS2 matured oocytes although 5meC of GV oocytes was intact.

3.6. Microarray analysis of GV oocytes after in vivo BPS2 treatment

As the altered 5meC levels in matured compared to GV oocytes suggested that upstream epigenetic factors may have been affected at the transcriptional level in immature BPS2 oocytes, we next evaluated the effect of BPS2 on gene expression in transcriptionally silenced GV oocytes. As the number of available samples was limited ($N = 4$ for both control and BPS2 groups), the results did not reach genome-wide significance after correcting for multiple comparisons. However, using arbitrary fold change cut-offs of > 1.5 and $P < 0.01$, 102 genes were up- (89) or downregulated (13) following BPS2 treatment (Fig. 6A and B). These results indicated that genes associated with cellular stress (in particular, *Cldn34b2*, *Gsdmc2,* and *Batf3*) were upregulated following BPS exposure. In contrast to our initial hypothesis, we observed alterations in factors related to embryonic development rather than epigenetic regulators (e.g., DNA methyl transferases and histone methyl transferases) in BPS-treated oocytes. In particular, *Ceacam10, Hist1h2af, Tma16*, and *Raptor* expression, which constitute markers of preimplantation and embryonic development, were upregulated. High *Tma16* and *Batf3* transcript levels appeared to be indicative of BPS-mediated changes during early embryonic development.

4. Discussion

BPS is currently utilized in many common consumer products, including cases in which legal prohibitions against BPA allow products containing BPS to be labelled 'BPA free'. Thus, considering that a recent biomonitoring study detected comparable instances of BPS-positivity to those for BPA [40,41] and BPS is more stable under heat and light than BPA [4], testing the biological effect of BPS is necessary to assess the risk to human health from BPS exposure [12].

For the present study, we chose an *in vivo* mouse model of acute BPS exposure. The tested doses were mostly lower than the established values for TDI (4 ng x g bw^{-1} day^{-1}) and much lower than the no-observed-adverse-effect level (NOAEL, 5 µg g bw^{-1} day^{-1}) and low-observed-adverse-effect level (LOAEL, 50 µg g bw^{-1} day^{-1}) for BPA

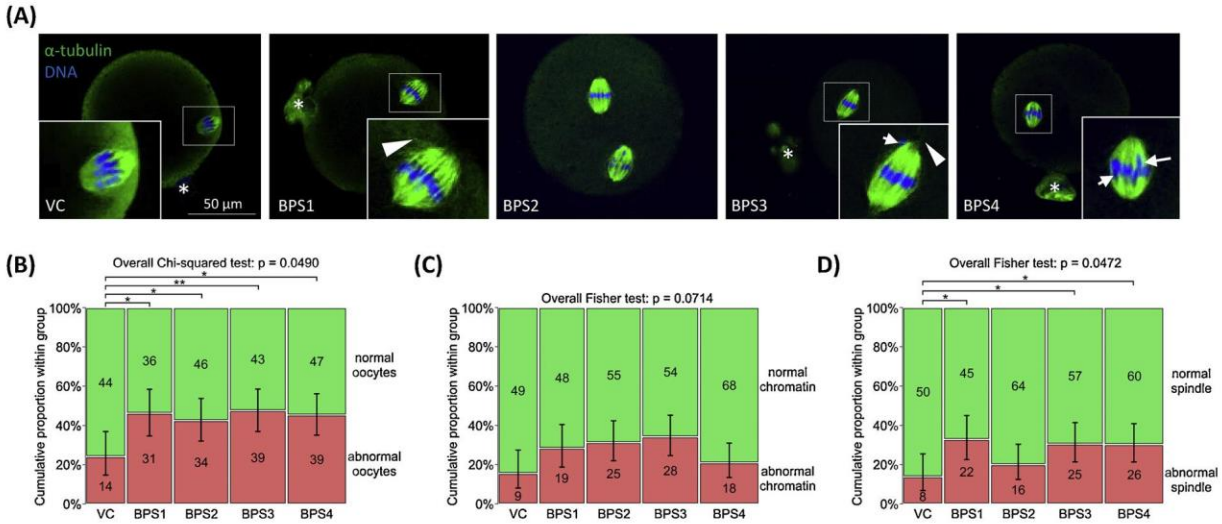

Fig. 2. Meiotic spindle and chromosomal alignment in BPS-exposed oocyte maturation. (A) Representative images of the chromatin and meiotic spindle of mature oocytes. Female mice were exposed to vehicle (VC, vehicle control) or different doses of BPS (BPS1–BPS4). Chromosome misalignment and spindle malformations were assessed based on DNA and α-tubulin staining, respectively. Arrows indicate individual chromatin aberrations, and arrowheads show unconjugated tubules on the spindle barrel and pole. (B–D) Chromatin and spindle malformations were identified in four independent experiments. The changes are expressed as the cumulative proportion of normal to abnormal oocytes, including oocytes with normal *vs.* abnormal chromatin and spindles. The values represent the number of oocytes in each experimental group. Error bars show 95 % confidence intervals for population proportions. Asterisks indicate statistical significance at $P < 0.05$ (*) and 0.01 (**) of pair-wise Fisher exact tests in a post-hoc role after overall significance testing as indicated.

[42]. However, a TDI for BPS has not yet been established, although very low doses of BPS have known risks [4]. Therefore, environmental BPS deserves rigorous attention, particularly in regard to human reproductive health.

We modelled population heterogeneity in the human population using a hormonally-unstimulated outbred mouse strain. To investigate the potential impact of BPS exposure on oocyte meiotic progression, we first evaluated oocyte number, the quality of immature oocytes (SN vs. NSN oocytes), re-initiation of meiotic maturation, and maturation rate. After noting no significant differences in these parameters, we focused on the oocyte quality in subsequent experiments and evaluated molecular markers of oocyte health.

First, the spindle apparatus was tested because it mediates chromosome alignment and segregation. The spindle also acts as the centre of physiologically asymmetric oocyte cytokinesis [35]. Defects in spindle assembly and abnormalities in chromosome alignment can result in meiotic progression failure and subsequently alter embryonic development consequent to fertilisation failure or aneuploidy [43,44]. Meiotic abnormalities in ova (e.g., unequal chromosome segregation) can cause changes in chromosome alignment and/or aneuploidy [45]. Moreover, presumed BPS-targeted cytoskeletal proteins and upstream factors are sensitive to BPS exposure [33]. In our experiments, abnormal oocyte levels were increased, including an increased incidence of abnormal chromosome alignment and/or spindle malformation, in all BPS-treated groups. Therefore, BPS causes spindle damage rather than chromatin misalignment. In particular, irregular spindle microtubule arrangement was the most frequent phenotype observed in our study, highlighting an oestrogen-like BPS effect similar to that reported in a previous study describing oestrogen-affected spindles [46].

Chromatin integrity was also assessed by quantifying double-strand DNA breaks. A deleterious effect of BPS on DNA integrity, indicated by increased TUNEL signal, was detected in BPS1, BPS2, and BPS4 groups.

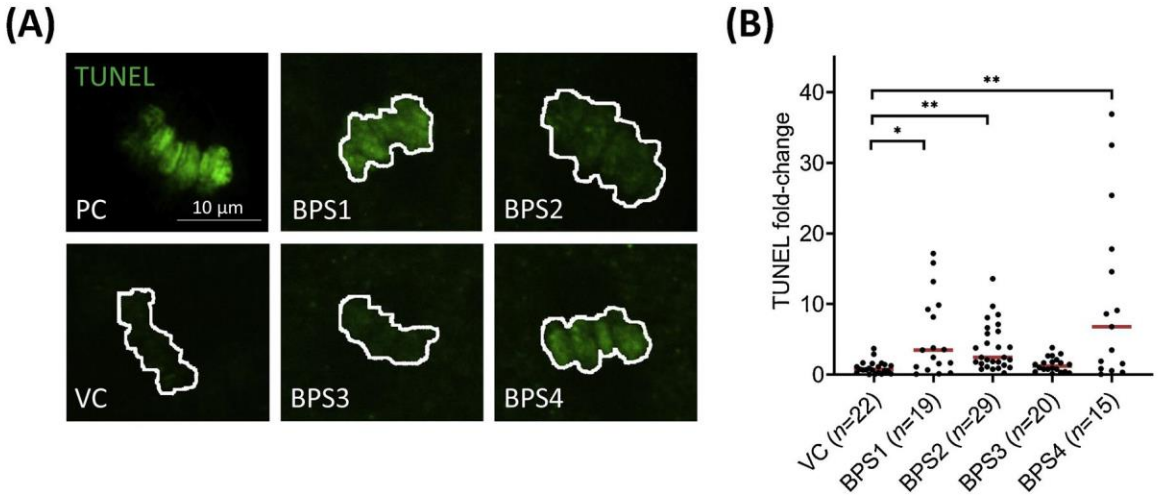

Fig. 3. DNA integrity in BPS-exposed oocyte maturation. (A) DNA integrity was evaluated using TUNEL assays. Pictures are representative of integrated density evaluated using ImageJ. Female mice were exposed to vehicle (VC, vehicle control) or different doses of BPS (BPS1–BPS4). PC: positive control. (B) Statistical differences were tested using a Kruskal-Wallis nonparametric test followed by Dunn's multiple comparison. Centre lines represent medians of individual values (*n*, the number of analysed oocytes, is noted in brackets for each experimental group). Asterisks indicate statistical significance at $P < 0.05$ (*) and 0.01 (**).

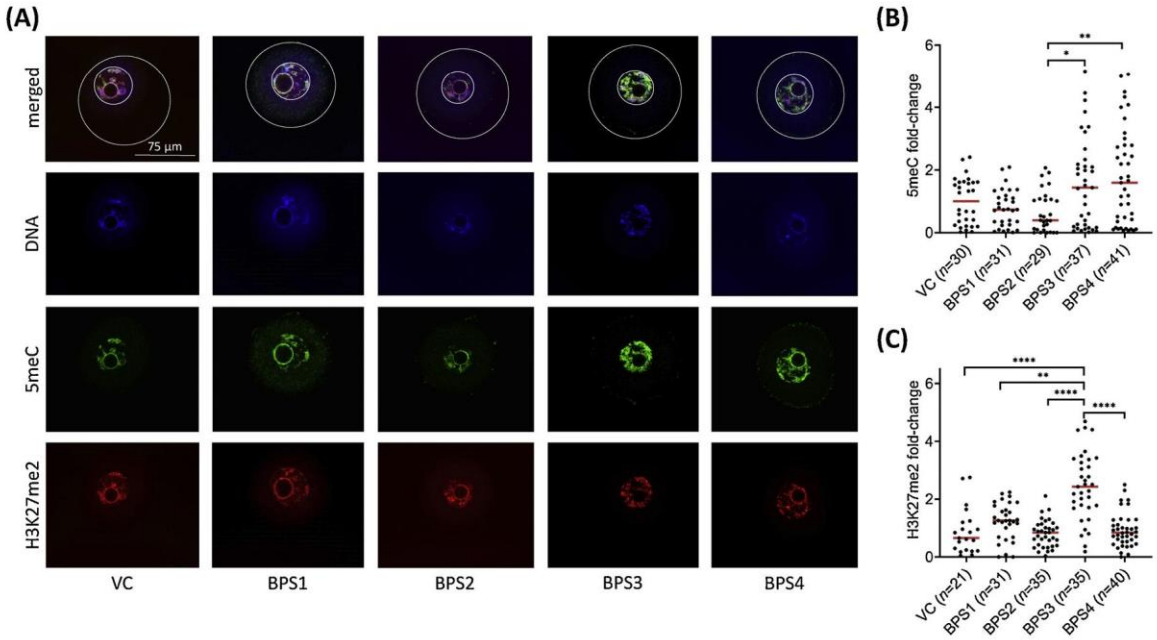

Fig. 4. Genome-wide epigenetic marks in immature germinal vesicle (GV) oocytes. (A) Representative images of 5meC (green) and H3K27me2 (red) chromatin-wide repressive marks in immature GV oocytes. Dashed and solid circles show the oocyte and GV border, respectively. (B, C) Integrated signal density for 5meC (B) and H3K27me2 (C) compared to that of the vehicle control group. Statistical differences were identified using the Kruskal–Wallis nonparametric test followed by Dunn's multiple comparison. Asterisks indicate statistical significance at $P < 0.05$ (*), 0.01 (**), or 0.0001 (****). Centre lines represent medians of individual values (*n*, the number of analysed oocytes, is noted in brackets for each experimental group).

These findings suggested that BPS functions as an endocrine disruptor, as observed in a recent endocrine disruptor study [47] and using a previously-established approach for DNA damage assessment [48]. This finding further underlines a non-monotonic curve in the response to BPS exposure, which was previously described as an endocrine disruption effect [5]. The effects of BPS as an endocrine disruptor support the findings in our reduced hormone stimulation donor mouse model.

In addition to cytoskeletal damage, endocrine disruptors also have a

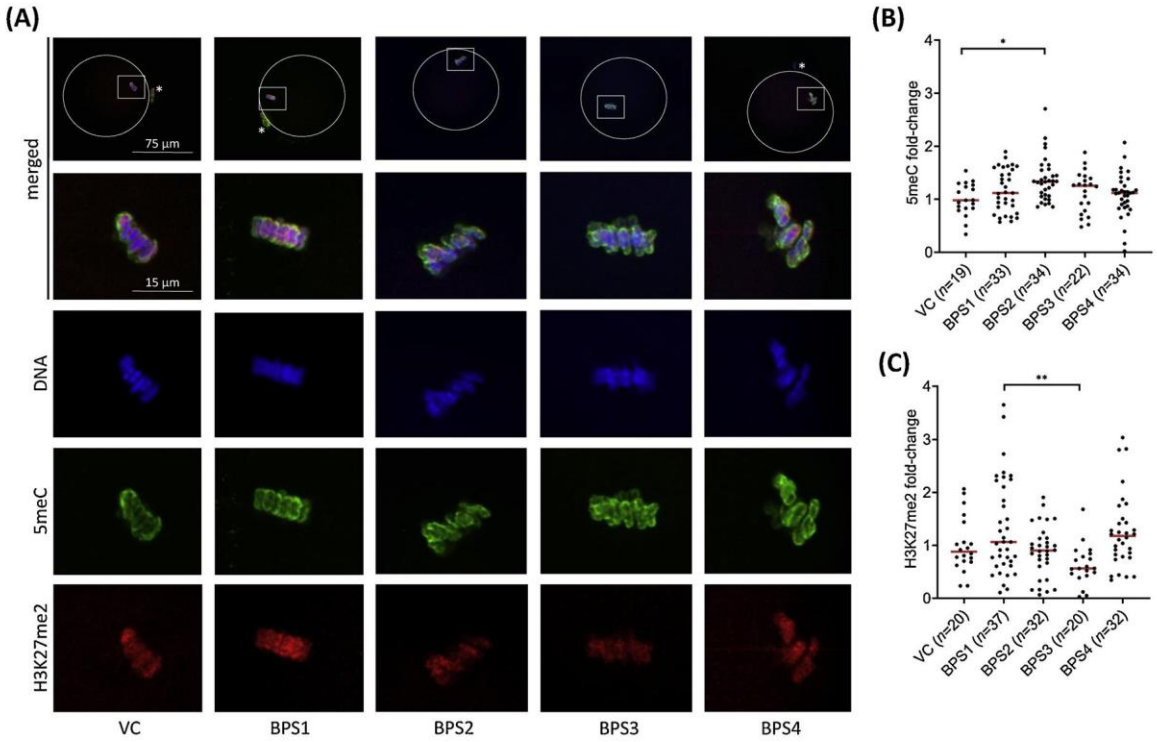

Fig. 5. Genome-wide epigenetic marks in mature MII oocytes. (A) Representative images of 5meC and H3K27me2 in mature MII oocytes. Dashed circle shows the oocyte border. The frame represents the emphasised area of the metaphase chromatin. The extruded polar body, which mark the mature oocyte, is indicated with an asterisk. (B, C) Integrated signal density of 5meC (B) and H3K27me2 (C) was compared to that of the vehicle control group. Statistical differences were tested using the Kruskal–Wallis nonparametric test, followed by Dunn's multiple comparison. Asterisks indicate statistical significance at $P < 0.05$ (*) or 0.001 (**). Centre lines represent medians of individual values (*n*, number of analysed oocytes, are noted in brackets for each experimental group).

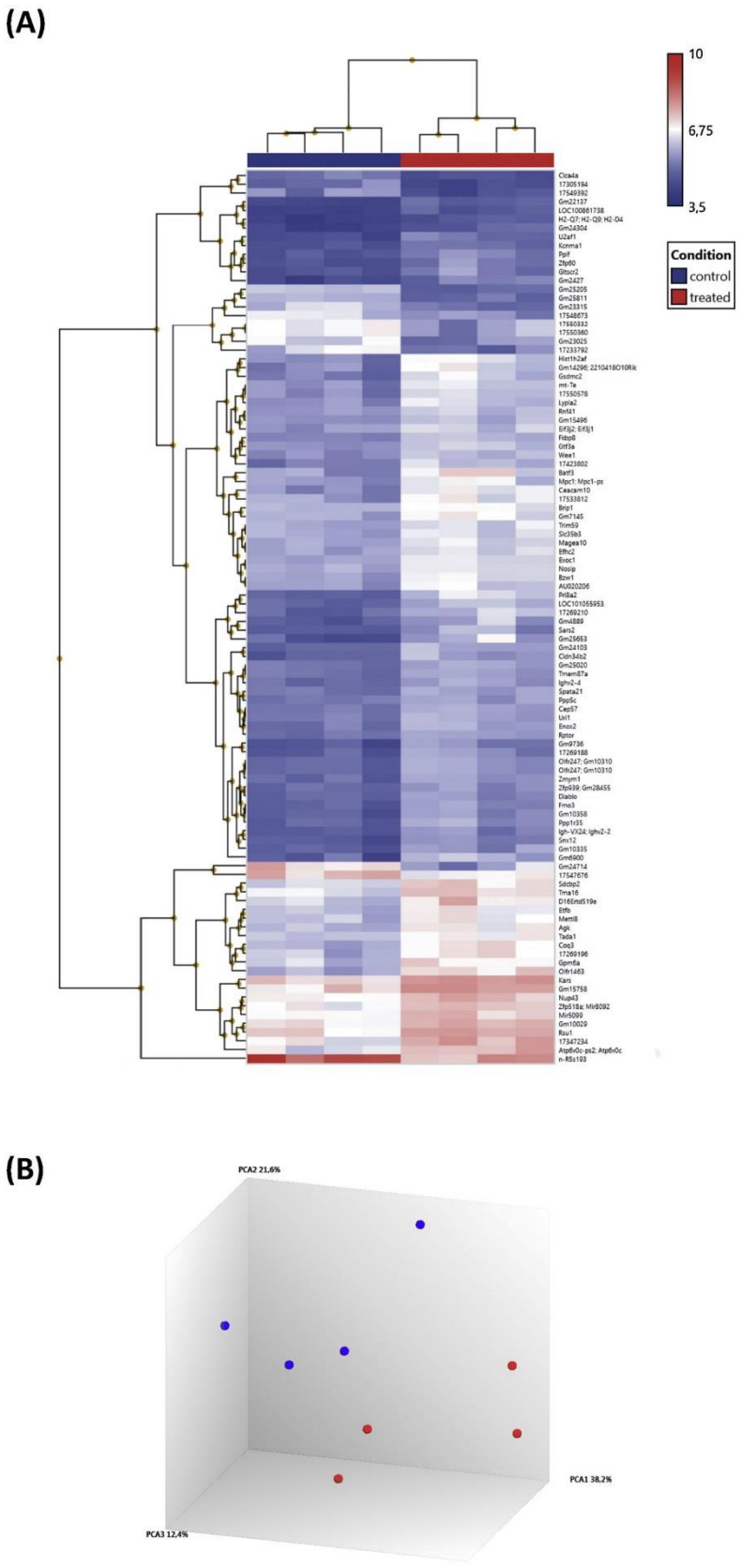

Fig. 6. Effect of BPS2 exposure on gene expression in mouse germinal vesicle (GV) oocytes. (A) mRNA expression heatmap in GV oocytes shows differences in gene expression between the vehicle control (control) and BPS2-treated oocytes. (B) Principal component analysis (PCA) and clustering of individual samples. Blue dots: control group, red dots: BPS2-treated group. Transcriptional profiling was performed using four pooled samples (equal to four independent experiments; 50 oocytes per sample from three animals) of the control and treated groups (i.e. n = 12 animals each).

well-defined epigenetic effect. We found that both genome-wide DNA and histone methylation (i.e., 5meC and H3K27me2), as genome-repressive marks, are vulnerable to disruption by BPS. In immature GV oocytes, H3K27 methylation was increased following BPS3 treatment. In contrast, 5meC levels increased in matured MII oocytes in the BPS2 group. Our observations thus support a previously-observed BPA effect on histone methylation in oocytes [49,50] and the BPS-induced H3K27me2 alterations [34]. However, the epigenetic effect varied with the mode of exposure (acute vs. chronic). The level of H3K27me2 increased following chronic exposure to BPS [34], whereas no effect was observed in present acute-exposure experiments. This discrepancy highlights the potential different targets of acute vs. chronic exposure, particularly preantral/antral follicles and earlier stages (i.e., primordial and/or primary follicles). Taken together, these findings suggest that BPS modulates epigenetically-driven gene expression, similar to BPA [51]. Furthermore, BPS-altered epigenomic programming can persist throughout the lifespan, thereby altering gene expression and possibly increasing disease susceptibility across generations [52].

Based on the findings that BPS2 oocytes also exhibit altered 5meC in matured MII oocytes, we considered GV ooplasmic factors that might underlie the epigenetic shift observed in MII oocytes. Moreover, the BPS2 dose of 0.1 ng BPS x g bw^{-1} day^{-1} is comparable to actual exposures [53] that exert detrimental effects on the cytoskeleton and DNA integrity. Because bisphenol alters epigenetic marks [54], we used microarray-based transcriptome analysis to profile the expression of genes related to genomic features and potential markers of oocyte quality of *in vivo*-exposed GV oocytes. We identified 102 genes as distinctly up- or downregulated following BPS treatment. In contrast to our hypothesis, rather than epigenetics-associated genes, the results suggested preferential biologically meaningful gene upregulation of preimplantation and embryonic development genes, such as *Raptor*, the central component of the mTOR complex 1, which is indispensable for oocytes and subsequent embryonic development [55]. Moreover, the two genes with the highest increased expression levels were *Tma16* and *Batf3*, which respectively encode a transcriptional machinery-associated protein and the basic leucine zipper transcription factor ATF-like 3, an RNA polymerase II proximal promoter sequence-specific DNA binding protein. Both genes regulate gene expression in early embryonic development. Therefore, fertilisation and early embryonic development appear to be highly sensitive to BPS, which may underlie the endocrine disruptor-induced reproductive failure.

In contrast, the transcriptome analysis results indicated that epigenetic factors (e.g., DNA methyl transferases, TET family dioxygenases) in the immature oocyte are likely affected by BPS in various ways other than through the targeting of transcription to exert an eventual epigenetic effect on matured oocyte chromatin. Such alternate BPS-sensitive mechanisms may include protein inactivation and/or inadequate post-translational modifications. Therefore, it is necessary to identify BPS molecular targets and interactions in gametes and early embryos, with the goal to protect human reproductive health. An obvious requirement also exists for further experiments focused on the fertilization and early embryonic development of BPS-affected oocytes, considering the qualitative changes between *in vitro*- and *in vivo*-matured oocytes, possible counteractions of bisphenols and hormonal stimulation (when assisted reproductive technologies are applied), and the developmental success of embryos following natural or *in vitro* fertilization.

In conclusion, acute exposure to very low BPS doses affects oocyte quality, as evinced by changes in the genome-wide epigenetic code and the transcriptional profile in immature GV oocytes. Moreover, BPS exposure significantly impacted the quality of GV oocytes, particularly spindle formation, DNA integrity, and epigenetic modifications, during meiotic maturation. Our observations therefore indicate that BPS is not a suitable replacement for BPA, and BPS intake should be limited.

Declaration of Competing Interest

None.

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Bisphenol S instead of bisphenol A: a story of reproductive disruption by regretable substitution – a review

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ABSTRACT: A range of substances that are released into the environment, foodstuffs and drinking water as a result of human activity were originally considered relatively harmless, and it was only later that their adverse effects were discovered. In general the use of such substances is currently restricted, and they are often replaced by other substances. This applies also in the case of a range of endocrine disruptors. These substances have the capacity to disturb the balance of physiological functions of the organism on the level of hormonal regulation, and their pleiotropic spectrum of effects is very difficult to predict. Endocrine disruptors include the currently intensively studied bisphenol A (BPA), a prevalent environmental pollutant and contaminant of both water and foodstuffs. BPA has a significantly negative impact on human health, particularly on the regulation mechanisms of reproduction, and influences fertility. The ever increasingly stringent restriction of the industrial production of BPA is leading to its replacement with analogues, primarily with bisphenol S (BPS), which is not subject to these restrictions and whose impacts on the regulation of reproduction have not yet been exhaustively studied. However, the limited number of studies at disposal indicates that BPS may be at least as harmful as BPA. There is therefore a potential danger that the replacement of BPA with BPS will become one of the cases of regrettable substitution, in which the newly used substances manifest similar or even worse negative effects than the substances which they have replaced. The objective of this review is to draw attention to ill-advised replacements of endocrine disruptors with substances whose effects are not yet tested, and which may represent the same risks for the environment, for the reproduction of males and females, and for human health as have been demonstrated in the case of the originally used substances.

Keywords: human health; environment; endocrine disruptor; reproduction; oocyte; sperm

INTRODUCTION

Many substances have been introduced into use with great hopes, only for it to be demonstrated earlier or later that they are harmful to the environment and/or human health. Notorious cases include the mass use of DDT as an insecticide (http://apps.who.int/iris/handle/10665/40018), thalidomide

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as a drug for pregnant women (McBride 1961), or more recently neonicotinoid insecticides used for the protection of fields against seed-destroying insects (Blacquiere et al. 2012). Substances whose negative effects on the environment or human health were detected only after a long period of use also include endocrine disruptors (Damstra et al. 2002).

The detection of the negative effects of abundantly used substances leads to a dramatic restriction of their use and their substitution with other substances. In a range of cases this brings about a genuine improvement. For example, chromated copper arsenate (CCA) used for wood preservation was demonstrated to be a substance with carcinogenic effects, and as a result was replaced with alkaline copper quaternary (ACQ). ACQ does not contain arsenic or chrome, and although it is just as effective as CCA against wood destroying arthropods, its impacts on the environment and human health are fundamentally less serious (Landrigan et al. 2004).

On the other hand, we have been witnesses to substitutions of harmful substances which have later been shown to be highly problematic. For example, 2,3-butanedione, which occurs naturally in butter, has been produced synthetically and added to foods in order to impart a buttery flavour. When it was demonstrated that 2,3-butanedione damaged lung tissue, it was replaced by 2,3-pentanedione, which however was subsequently proven to have similar negative effects on lung tissue as 2,3-butanedione (Hubbs et al. 2012). There are far more similar examples of "regrettable substitutions" (Fahrenkamp-Uppenbrink 2015; Zimmerman and Anastas 2015). In these cases, negative impacts on reproduction are often subsequently detected. For example, in the case of pyrethroids, which replaced older insecticide agents such as organocholorines, organophosphates or carbamates, and which were considered harmless to mammals, negative impacts were demonstrated on the maturation of mammal oocytes (Petr et al. 2013).

From the perspective of reproductive risks, the substitution of bisphenol A (BPA), a widely used component of plastics and many other materials, with its analogue bisphenol S (BPS) appears to be potentially problematic. BPA has been proven to be a strong endocrine disruptor, and its use has been restricted. Many products are sold with a "BPA-free" guarantee. Because BPA is substituted in a range of cases by BPS, these products are not however "bisphenol-free" (Glausiusz 2014), and their use may be linked to significant reproductive risks. The aim of this review is to point to the replacement of BPA by BPS as a "regrettable substitution".

Endocrine disruptors

A less harmful substitute is currently searched for a number of substances that had previously been considered safe from a toxicological perspective and finally appeared to exert various negative effects on health. This category of compounds includes substances referred to summarily as endocrine disruptors (Clayton 2011). According to the US Environmental Protection Agency, endocrine disrupting chemicals (EDCs) are defined as "exogenous agent(s) that interfere(s) in synthesis, secretion, transport, metabolism, binding action, or elimination of natural blood-borne hormones that are present in the body and are responsible for homeostasis, reproduction, and developmental processes" (Diamanti-Kandarakis et al. 2009).

EDCs manifest a range of particular properties. Their hormone-like effects may be suppressed or may fade away entirely in the case that the concentration of EDCs is higher than the physiological level of their hormonal counterpart. This ability of agents to attain paradoxically stronger effects in low doses than in high ones (vom Saal and Welshons 2005) is termed the "low dose effect" (Grasselli et al. 2010; Vandenberg et al. 2012). The low dose hypothesis posits that exogenous chemicals that interact with hormone action can do so in a quite specific manner. In accordance with that, mentioned traditional toxicological endpoints are not capable to preclude adverse outcome, as EDCs act with dose responses, that are nonlinear and potentially non-monotonic (Vandenberg et al. 2012). In the case the relationship between dose and response is nonlinear, any prediction is even more complex. Therefore, the low dose definition was extended by the effects of non monotonic response curves. The mechanisms responsible for the non-linear effects are described in detail (Vandenberg et al. 2012), usually in connection with an interaction between a ligand (hormone or EDC) and a hormone receptor (Vandenberg 2014).

Non-linear dose-response patterns are commonly observed with endogenous and synthetic agonists (e.g. numerous drugs, hormones, peptides) that activate and inhibit receptor-mediated signal pathways that affect various biological functions

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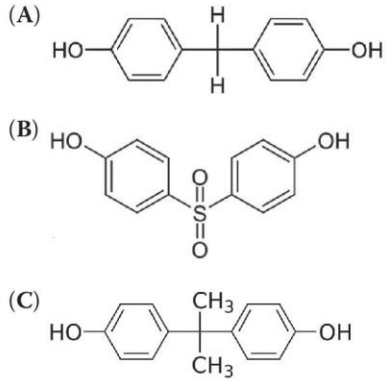

Figure 1. Chemical structure of bisphenol A (**A**), bisphenol S (**B**), bisphenol F (**C**)

(Calabrese and Baldwin 2001; Calabrese 2005). However, EDCs can also produce non monotonic dose responses in which the slope of the curve changes sign over the course of the dose-response (www.who.int/ceh/publications/endocrine/en/index.html) and low dose effects are described for the majority of EDCs (Birnbaum 2012; Vandenberg et al. 2012, 2013; Zoeller et al. 2012; Bergman et al. 2013).

The concept of endocrine-disrupting chemicals was proposed after these compounds had been observed to affect various reproductive functions in wildlife and humans (Colborn et al. 1993). The influence of several EDCs was demonstrated on the course of development of male gametes, sperm (Li et al. 2011; Knez et al. 2014) and female gametes, oocytes, as well as embryonic development of males and females (Mok-Lin et al. 2010; Xiao et al. 2011). Moreover, the effect of EDCs on the reproduction of adult individuals, including transgenerational inheritance, has been described (Susiarjo et al. 2015; Ziv-Gal et al. 2015). Therefore, reproductive functions represent crucial targets of the EDCs' negative effects. Recently intensively studied EDCs, interfering with the regulation of physiological reproductive processes, include bisphenols, a family of chemical compounds with two hydroxyphenyl functional groups (Figure 1).

Bisphenol A

An example of a widely used substance, in which endocrine-disrupting properties were detected only later, is bisphenol A (BPA, 4,4'-(propane-2,2-diyl)diphenol) (Vandenberg et al. 2009). BPA was first synthesized in 1891, and as early as in 1936 it was demonstrated that it imitates the activity of the hormone estradiol (Dodds and Lawson 1936). Despite a very strong estrogen activity, BPA has been commercially used since 1957, and despite the fact that its endocrine-disrupting activity was discovered (Krishnan et al. 1993), BPA has become a high production volume chemical (Wang et al. 2012). Worldwide annual production, which in the case of BPA reached 4.6 million t in 2012, is constantly increasing. Its production was estimated at 5.4 million t in 2015 (Merchant Research & Consulting, http://mcgroup.co.uk/researches/bisphenol-a-bpa).

BPA is present especially in polycarbonate plastics, epoxide resins, and several paper products (Ehrlich et al. 2014), and as a result it is used in a variety of commonly used consumer products such as thermal recipes, cosmetics, dental materials, medicinal tubes, utensils, toys, baby feeding bottles and dummies, etc. Heat, UV radiation, alkaline treatment or intensive washing causes a release of BPA monomer. It is estimated that the worldwide release of BPA into the environment is almost half million kg per year (Mileva et al. 2014).

BPA is released into the environment either directly from chemical, plastic coating, and staining manufacturers, from paper or material recycling companies, foundries which use BPA in casting sand, or indirectly leaching from plastic, paper, and waste in landfills (Yang et al. 2015). BPA passes into foodstuffs or water directly from the lining of food and beverage cans, where it is used as an ingredient in the plastic used to protect the food from direct contact with the can (Goodson et al. 2002; Vandenberg et al. 2009). The main path of human exposure is the consumption of such contaminated foodstuffs, drinking water or via dermal contact with thermal paper and cosmetics or inhalation (Miyamoto and Kotake 2005; Huang et al. 2012).

It is therefore not surprising that a range of studies have now demonstrated the presence of BPA in human tissue. Levels of BPA have been tested in various populations worldwide, and the presence of BPA was demonstrated in 92.6% of Americans (Wetherill et al. 2007) and 90% of Canadians (Bushnik et al. 2010). Levels of BPA have been demonstrated in various biological matrices, most frequently in urine (Casas et al. 2013; Salgueiro-Gonzalez et al. 2015), but also in blood serum. Within the human reproductive system, levels of BPA have been confirmed for example in testicle tissue, seminal plasma (Manfo et al. 2014), in ovarian follicular fluid (Ikezuki et al. 2002), mother's

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Table 1. Bisphenol A (BPA) levels in human fluids

Sample	Level of BPA	References
Blood (ng/ml)	12.4–14.4	Bushnik et al. (2010)
Maternal blood (ng/ml)	0.63–14.36	Yamada et al. (2002)
Fetal blood (ng/ml)	0.2–9.2	Schonfelder et al. (2002)
Urine (ng/ml)	0.02–21.0	Liao et al. (2012c)
Saliva (ng/ml)	0.3	Joskow et al. (2006)
Follicular fluid (ng/ml)	2.4 ± 0.8	Ikezuki et al. (2002)
Amniotic fluid (ng/ml)	1.1–8.3	Ikezuki et al. (2002)
Placental tissue (ng/g)	1.0–104.9	Schonfelder et al. (2002)
Breast milk (ng/ml)	0.5–1.3	Mendonca et al. (2014)
Semen plasma (pg/ml)	66 (fertile men) 132–179 (infertile men)	Vitku et al. (2015)

milk, fetal plasma (Shonfelder et al. 2002), amniotic fluid (Yamada et al. 2002; Edlow et al. 2012), and the placenta (Jimenez-Diaz et al. 2010; Cao et al. 2012) (Table 1). Several studies have demonstrated a direct correlation between exposure of the mother and the BPA level of the fetus (Ikezuki et al. 2002; Kuruto-Niwa et al. 2007). BPA may permeate the placenta and thus influence the development of the fetus (Edlow et al. 2012; Corbel et al. 2014). Newborns may then be further exposed to the effect of BPA during breastfeeding due to the presence of BPA in mother's milk (Mendonca et al. 2014).

The effects of BPA on humans are dependent not only on the dose, but also on the window of exposure. Exposure to BPA in the prenatal and neonatal period probably affects the human organism in the most receptive period (Fernandez et al. 2014).

Mechanism of BPA action

A typical feature of endocrine disruptors is their wide spectrum of outcomes (Figure 2). Combination of their action in various target systems in the organism is one of causes of their non-linear effects. In this respect, BPA acts as a typical endocrine disruptor with multi-level impacts (Khan and Ahmed 2015). Nongenomic effects of BPA have been described, thus influencing cellular signalling

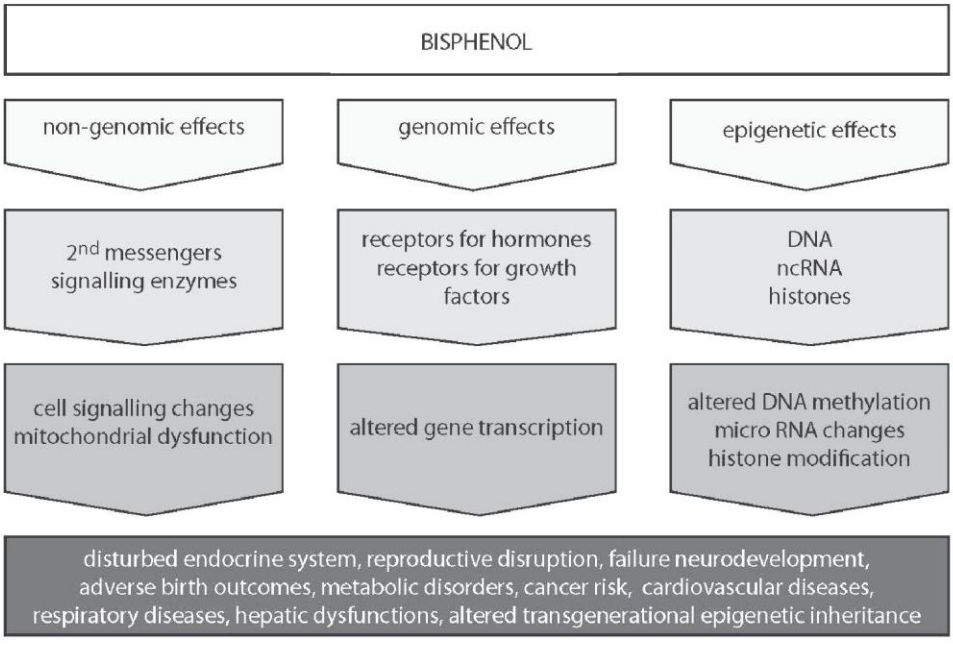

Figure 2. Possible mechanisms of bisphenol action and its potential impact on human health

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(Nakagawa and Tayama 2000), as well as genomic, which affect transcription regulation (Trapphoff et al. 2013), and also epigenetic, responsible for the methylation and acetylation of DNA and core histones (Bromer et al. 2010). It is precisely pronounced estrogen activity of BPA *in vitro* (vom Saal et al. 2007; Wetherill et al. 2007) and *in vivo* that contributes to its immense potential to afflict the hormonal system and act as an endocrine disruptor.

BPA inhibits the activity of natural endogenous estrogens and thus disrupts estrogen nuclear hormone receptor action (Kitamura et al. 2005; Wetherill et al. 2007; Grignard et al. 2012). BPA affects hormonal homeostasis, for example through bonding to the classic nuclear estrogen receptors α, β, γ (ERα, ERβ, ERγ), where it manifests a combination of agonistic and/or antagonistic actions in dependence on the target tissue, cell types, ER subtypes, and differential cofactors recruited by ER-ligand complexes (Kurosawa et al. 2002). BPA also bonds to non-classical membrane ERs and causes activation of the nuclear receptor gamma (Takayanagi et al. 2006; Matsushima et al. 2007).

BPA has been identified as an antagonist of androgen receptors (Kitamura et al. 2005; Wetherill et al. 2007; Vinggaard et al. 2008; Molina-Molina et al. 2013). Its anti-androgenic activity has been documented in several studies, but with changing values of the maximum inhibition concentration (Xu et al. 2005; Bonefeld-Jorgensen et al. 2007). In contrast with other known androgen receptor antagonists, BPA inhibits the effective nuclear translocation of the androgen receptors, and disrupts their function by means of a number of mechanisms (Teng et al. 2013). The endocrine-related BPA action mechanism also involves a reduction of aromatase expression (Zhang et al. 2011; Chen et al. 2014) and a decrease in aromatase activity *in vitro* (Bonefeld-Jorgensen et al. 2007). Within this context, it is of interest that a decline in the synthesis of testosterone and estradiol *in vivo* has been documented following exposure to BPA (Akingbemi et al. 2004).

The epigenetic mechanisms of the effect of BPA include the alteration of certain DNA methylation samples (Dolinoy et al. 2007; Susiarjo et al. 2013). Prenatal exposure to BPA alters the expression of genes coding individual subtypes of ERs in a sex- and brain region-specific manner (Kundakovic et al. 2013) and disrupts the normal development of the placenta (Susiarjo et al. 2013). As a result, it is possible that BPA predetermines the response to steroid hormones in the very early phase of development (Wilson and Sengoku 2013). It has been documented that BPA also disrupts the gene expression of the regulating factors that control the stability and flexibility of epigenetic regulation, and as a result has an adverse influence on the development of functions of the controlling organ of hormonal regulation, the hypothalamus (Warita et al. 2013). The impacts of these changes have transgenerational effects (Manikkam et al. 2013).

Further demonstrated actions of BPA in the organism include the bonding to the glucuronide receptor, suppression of the transcription receptor of the thyroid hormone, reduction of the transport of cholesterol via the mitochondrial membrane, increase of oxidation of fatty acids, stimulation of prolactin release (Machtinger and Orvieto 2014) or an agonistic effect on the human pregnane X receptor (Sui et al. 2012).

BPA and human health

With such a wide spectrum of effects, it is evident that BPA has a negative influence on human health. Frequently discussed themes include the possible association of BPA for example with obesity (Trasande et al. 2012), diabetes (Lang et al. 2008), neurobehavioural disorders (Jasarevic et al. 2011), cancer (Jenkins et al. 2011), hepatic (Peyre et al. 2014) and cardiovascular diseases, hypertension, and disorders of the thyroid gland function (Rochester 2013; Wang et al. 2013).

Especially in the area of reproduction in both animal models and in humans, a wide range of negative influences of BPA have been observed (Kwintkiewicz et al. 2010; Trapphoff et al. 2013; Zhang et al. 2014). BPA has varied and complex mechanisms of action that may interfere with normal reproductive development and functions. In both males and females, BPA interferes with hormonal regulation and influences the hypothalamic–pituitary–gonadal axis on all levels (Navarro et al. 2009; Patisaul et al. 2009; Xi et al. 2011).

Influences of BPA on reproduction of males

As a rule, endocrine-disrupting substances have pronounced impacts on the reproduction of both sexes. Several studies have shown detrimental effects of BPA on spermatogenesis and semen quality in fishes. The number of mature and im-

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mature spermatozoa was decreased and increased, respectively (Sohoni et al. 2001) and also the sperm motility and concentration were reduced (Lahnsteiner et al. 2005). There is a large evidence that BPA can induce sex reversal from male to female in aquatic animals. Changes in sex ratio were observed at zebrafish during embryonic development (Drastichova et al. 2005) and *Xenopus* larvae through metamorphosis (Kloas et al. 1999).

Experimental studies on the effects of BPA on the reproduction of male rodents have revealed an adverse influence on the development of testes (Vrooman et al. 2015) and on the spermatogenesis of adult individuals following prenatal *in utero* or early postnatal exposure. Exposure to BPA during the period of development of the testes is frequently linked to a range of negative effects in adult testes, e.g. decreased levels of testicular testosterone, decreased weights of the epididymis and seminal vesicles, a decrease in daily sperm production per gram testis, and increased weights of the prostate and preputial (Richter et al. 2007). Vrooman et al. (2015), with the help of transplantation of spermatogonia from the testes of mice exposed to the action of BPA into mice which were not exposed, demonstrated permanent damage to spermatogenesis. The influence of the exposure of adult rodents to BPA on the quality of sperm was also studied (Peretz et al. 2014).

Despite the differences in the experimental designs used, certain findings appear repeatedly, especially reduction in the number of sperm, reduction in the motility of sperm, increased amount of apoptotic cells in the seminiferous tubules, changes in the levels of hormones and steroid enzymes, and damage to the DNA of sperm (Peretz et al. 2014).

Contemporary studies confirm that rodents are not relevant for predicting the effect of low BPA concentrations on the endocrine function of human fetal testis (N'Tumba-Byn et al. 2012). In a comparative study by Maamar et al. (2015), the influence of BPA was studied both on rats and on human fetal testes, and it was determined that in both cases BPA had dose-dependent anti-androgenic effects. Nevertheless, the authors urge caution in interpreting the results obtained on rodents and their application in human medicine (Maamar et al. 2015).

Unfortunately, there is only a limited number of studies that have observed the influence of exposure to BPA on the quality of sperm in adult humans. In men exposed to BPA in the workplace and patients in reproduction centres, a higher level of BPA in urine was linked to a lower number, concentration, and motility of sperm (Knez et al. 2014; Lassen et al. 2014). Nevertheless, in a study conducted by Mendiola et al. (2010) on fertile men, the concentration of BPA in urine did not correlate with changes in semen parameters, despite the fact that a significant correlation was observed between the level of BPA in urine and the volume of seminal plasma or markers of free testosterone (Mendiola et al. 2010).

The following cohort study examined the relationship between the concentration of BPA in urine and the level of reproductive hormones and semen in a group of 308 young healthy men. It was determined that the concentration of BPA strictly correlates with higher levels of selected circulating reproductive hormones and reduced motility of sperm. The results indicated that the exposure to BPA on the level of environment has an anti-androgenic and/or anti-estrogenic effect due to the effect of BPA on the level of receptors. The anti-estrogenic effect on the level of the epididymis also explains the determined low mobility of the sperm (Lassen et al. 2014).

Influences of BPA on reproduction of females

BPA markedly influences not only the reproduction of males, but also the reproduction of females. In both *in vitro* and *in vivo* studies, the influence of BPA has been demonstrated on fertility, function of the womb i.e. formation of benign and malignant lesions (Newbold et al. 2009), disruption apoptosis of the uterine epithelium during estrus (Mendoza-Rodriguez et al. 2011), function of ovaries and quality of oocytes (Peretz et al. 2014), and defective folliculogenesis (Santamaria et al. 2016). In females it is precisely the ovaries that are the key organ responsible for reproductive and endocrine functions, and BPA is frequently indicated as an ovarian toxicant. BPA afflicts not only the overall morphology and weight of the ovaries (Suzuki et al. 2002; Santamaria et al. 2016) but also demonstrably reduces the quality of oocytes in both animal and human models (Machtinger and Orvieto 2014).

During the course of the maturation of mouse oocytes *in vitro* following treatment with BPA, changes were documented in the configuration of the meiotic spindle resulting in errors in chromosome segregation and hyperploidy frequencies in mouse

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oocytes (Hunt et al. 2003). Similary, it was reported that BPA exposure altered chromosome and spindle organization which resulted in hyperploidy of mouse oocytes during meiosis (Can et al. 2005) and it was also demonstrated that low BPA doses are related with aberration during meiotic prophase, including increased incidence of recombination (Susiarjo et al. 2007) and failure formation of primordial follicle by inhibiting meiotic progression of oocytes (Zhang et al. 2012). In contrast, Eichenlaub-Ritter and her colleagues found no evidence that low BPA doses increased hyperploidy at meiosis II. On the other hand they observed cell cycle delay and meiotic spindle abnormalities, changes in the distribution of pericentriolar material and chromosome alignment (Eichenlaub-Ritter et al. 2008). Exposure of mice, from mid-gestation to birth, causes synaptic abnormalities in oocytes and an increased amount of recombination between homologous chromosomes. It is also of interest that identical effects have been observed in homozygous mice with an intentionally disrupted gene coding the ERβ. In mouse oocytes, epigenetic changes have also been documented following cultivation of follicles in the presence of BPA, in which a disruption of the configuration of chromosomes took place, as well as disorders of meiosis caused by faulty genomic imprinting and altered posttranslational modification of histones (Trapphoff et al. 2013). Chronic exposure of oocytes was linked to an increased incidence of aberrant metaphases II and prematurely segregated chromatids (Pacchierotti et al. 2008).

Bovine oocytes cultivated in the presence of BPA have also manifested disorders of the meiotic spindle and the chromosomal configuration (Ferris et al. 2015). In Barbary Macaques, negative effects of BPA have been demonstrated in various stages of the oogenesis of developing ovaries. Oocytes in the prophase of meiosis and in fetal ovaries exhibited an increased number of recombination, and an increased number of abnormally formed follicles containing multiple oocytes was recorded in perinatal ovaries (Hunt et al. 2012).

Similary as in the aforementioned studies on rodents, cattle, and primates, an increased number of crossing over and degenerations in oocytes have been determined also in human oocytes cultivated *in vitro* in the presence of BPA (Brieno-Enriquez et al. 2011). In connected studies it has been demonstrated that the exposure of human oocytes to BPA is linked to up-regulation of genes involved in meiotic processes connected to double strand breaks repair progression (Brieno-Enriquez et al. 2012). A non-linear response to BPA doses on the incidence of MII oocytes with aligned chromosomes has also been determined (Machtinger et al. 2013). The changes which have been recorded in the development of oocytes exposed to bisphenol may lead to disorders in the development of embryos, fetal loss or genetic disorders (Rama Raju et al. 2007; Ye et al. 2007; Tomari et al. 2011). The result of maternal exposure to BPA may be the disruption of the entire oogenesis in the developing ovary (Susiarjo et al. 2007).

A number of cohort studies have been focused on groups of persons who undergo treatment for infertility through *in vitro* fertilization (IVF). The measured levels of BPA in these persons were examined in connection with the ovarian response, quality of embryos and implantation. A reduced ovarian response was linked to a reduced success rate of IVF (Mok-Lin et al. 2010). BPA also disrupted embryonal development of fish via delay hatching, yolk reabsorption, and larval growth of trouts (Aluru et al. 2010), moreover lethality in zebrafish larvae increased (Chan and Chan 2012).

There is only a limited number of studies which have observed the effects of BPA on the development and quality of mammalian blastocysts. Failure of embryonic development to mouse blastocyst stage has been demonstrated after exposure of females to BPA (Xiao et al. 2011). Disorder of implantation of mouse blastocysts was also demonstrated by Borman et al. (2015).

In human, Bloom et al. (2011) state a correlation between the concentration of BPA in the urine of men, though not in women, and a decline in the quality of embryos generated by IVF. By contrast, in a study performed by Knez et al. (2014), which confirms changes to the semen quality of men with a determined environmental level of BPA, undisrupted development of embryos into blastocysts is described. As against this finding, in women who have undergone IVF, a correlation has been demonstrated between the concentration of BPA in urine and a change to the formation of blastocysts, though a reduced quality of embryos was not recorded (Ehrlich et al. 2012).

The advent of BPS

The above-stated facts led to the necessity for stringent regulation of the use of BPA, and in a

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range of cases its substitution with another chemical. On the basis of the effects on human health and reproduction demonstrated with the help of standardized toxicological testing procedures, government agencies in the United States (the US Environmental Protection Agency, USEPA), Canada (Health Canada), and Europe (the European Food Safety Authority, EFSA) have established tolerable daily intake levels, ranging from 25 to 50 μg BPA/kg of body weight (BW) per day (Rochester 2013). With regard to the fact that several studies have demonstrated BPA low dose effects (Vandenberg et al. 2012), and that this possibility is unfortunately not taken into account in the approach of "traditional" toxicological studies, in which low doses are not generally subjected to examination (Vandenberg et al. 2012; Rochester 2013), scientists have expressed concerns that the "safe" cut-off set for BPA is too high (vom Saal and Hughes 2005). In 2010 the Canadian government prohibited the import, sale, and advertisement of baby feeding bottles containing BPA. The European Union responded with a prohibition of the manufacture of baby feeding bottles with BPA, which was passed in 2011 (Commission Directive 2011). The Food and Drug Administration (FDA) has indicated BPA as a "chemical of concern", and in July 2012 a blanket prohibition of BPA in baby feeding bottles and sippy cups was recommended (FDA 2011). However, new data and refined methodologies have led EFSA experts to considerably reduce the safe level of BPA from 50 μg/kg of BW/day to 4 μg/kg of BW/day (EFSA 2014).

With regard to these restrictions and societal pressures, manufacturers of plastics are now forced to seek an alternative product which can replace BPA. It is in the interest of chemical concerns that the substitute which replaces BPA is inert or at least far less toxic than BPA. Nevertheless, new chemicals introduced onto the market are frequently untested, and may be equally or more harmful than the originals, which are ultimately termed "regrettable substitutions" (Rochester and Bolden 2015), as has been the case of a number of perfluorinated chemicals (Howard 2014), pesticides (Coggon 2002), and self-extinguishing compounds (Bergman et al. 2012). Manufacturers seeking BPA alternatives have turned primarily to bisphenol S (BPS, 4,4'-sulfonyldiphenol) (see Figure 1), a structural analogue of BPA, to produce "BPA-free" products (Grignard et al. 2012; Barrett 2013). BPS is chemically more stable, worse in terms of biodegradability than BPA, and shows better dermal penetration than BPA (Ike et al. 2006; Danzl et al. 2009; Liao et al. 2012a, b). It is disconcerting that these properties may lead to a longer or higher body burden or bioavailability of BPS versus BPA (Helies-Toussaint et al. 2014). For these reasons, too, at present the replacement of BPA with BPS is considered a "regrettable substitution" (Fahrenkamp-Uppenbrink 2015; Zimmerman and Anastas 2015). With regard to the increase in production of BPS and the indispensability of bisphenols in the production of plastics, it is unfortunately possible to expect the same widespread use of BPS as in the case of BPA (Liao et al. 2012c). Now the presence of BPS can be expected in almost all the consumer goods here in which BPA was initially used (Mathew et al. 2014), for example as a wash fastening agent in clearing products, an electroplanting solvent, and a constituent of phenolic resins (Rochester and Bolden 2015).

One of the major industries that have replaced BPA due its high occurrence (~3–22 g/kg) is that of thermal paper (Mathew et al. 2014). In the USA, Korea, Vietnam, Japan, and China (Liao et al. 2012c), BPS has been detected in several different "BPA free" paper products, including receipts and paper money (Liao et al. 2012a). The presence of BPS has been determined in tinned foodstuffs (Vinas et al. 2010). The occurrence of BPS has also been determined in indoor dust (Liao et al. 2012b), in fluvial water (Ike et al. 2006), surface water, and waste waters (Song et al. 2014) (Table 2).

The main pathway to the human body is dermal, dust ingestion, and dietary exposures (Liao et al. 2012b). Unfortunately, for example thermal paper carries BPS into all recycled paper products, making dermal exposure inevitable. Massive exposure of the population to the effects of environmental BPS has been demonstrated in a number of different countries. Within the range of 0.02–21 ng/ml (0.8–84nM) it has been detected in human urine samples originating from seven Asian countries and the USA (Liao et al. 2012a) in 81% of analyzed samples. In the following study the presence of BPS in urine was demonstrated in residents living near a manufacturing plant in south China in a concentration of 0.029 ng/ml (Yang et al. 2015).

Biological effects of BPS

Although nowhere near as much information is available about BPS as about the endocrine-

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Table 2. Bisphenol S (BPS) levels in the personal care products and environment

Sample	Level of BPS	References
Canned food (ng/g)	8.9–17	Vinas et al. (2010)
Thermal paper (mg/g)	0.0000138–22.0	Liao et al. (2012c)
Tickets (μg/g)	0.183–5.93	Liao et al. (2012c)
Currency bills (μg/g)	0.00–6.26	Liao et al. (2012c)
Other paper product types (μg/g)	0.00–8.38	Liao et al. (2012c)
Indoor dust (μg/g)	0.34	Liao et al. (2012b)
Municipal sawage sludge (ng/g dry weight)	0.17–110.00	Song et al. (2014)
River water (ng/l)	0.29–18.99	Yang et al. (2014)

disrupting effects of BPS, the substitution of BPA with BPS is raising concerns. The limited number of studies available at the present time, dealing with the biological interactions of BPS with the organism, indicate that BPS is also capable of imitating properties of hormones, interacting with ER (Delfosse et al. 2012; Rosenmai et al. 2014; Le Fol et al. 2015), and direct binding to nuclear ERs (Yamasaki et al. 2004) and serum albumins (Mathew et al. 2014) has been confirmed.

Some *in vitro* studies have demonstrated a weaker estrogen activity of BPS than the activity manifested by estradiol (Kuruto-Niwa et al. 2010; Grignard et al. 2012; Molina-Molina et al. 2013; Rochester and Bolden 2015). By contrast, a study conducted by Vinas and Watson (2013a, b) demonstrated the same or higher estrogen effectiveness than estradiol, BPS was capable of stimulating the membrane receptor pathways ordinarily up-regulated by estradiol. After exposure to BPS there are also changes in the expression of aromatase, the key enzyme in the synthesis of estradiol (Kinch et al. 2015).

Like in the case of BPA, the androgenic activity of BPS was confirmed (Kitamura et al. 2005), and subsequently its anti-androgenic activity as well (Molina-Molina et al. 2013). These observations *in vitro* have also been confirmed by *in vivo* studies. Chen et al. (2002) described acute toxicity of BPS in *Daphnia magna* and at the same time also demonstrated estrogen activity of BPS *in vitro*. Yamasaki et al. (2004) documented estrogen activity of BPS *in vivo* in rats with the assistance of postnatal exposure to BPS, which in both low and high doses induced the growth of the womb (Owens and Ashby 2002). An *in vivo* study on the effect of BPS in zebrafish documented not only changes in the mass of the gonads and plasmatic levels of estrogen and testosterone, but also a marked disruption of reproduction. The study of Qiu and colleagues evaluated the impact of BPA and BPS on the reproductive neuroendocrine system during zebrafish embryonic development, and explored potential mechanisms of action associated with ER, thyroid hormone receptor, and enzyme aromatase pathways. All of these pathways were necessary to observe the full effects of BPS on the changes in gene expression in the reproductive neuroendocrine axis (Qiu et al. 2016). These data were substantiated by a decrease in egg production and hatchability and an increasing number of embryo malformations (Ji et al. 2013). These observations were later extended upon by increased time to hatch, reduced number of sperm, increasing number of female to male ratio, and changes in the levels of testosterone, estradiol, and vitellogenin (Naderi et al. 2014). In further experiments provided in cell cultures it has been demonstrated that BPS acts cytotoxically, genotoxically (Lee et al. 2013), and mutagenically (Fic et al. 2013).

The reason for these negative effects may be for example binding to serum albumins or DNA damage and subsequent influencing of several signal cascades anywhere within the organism (Lee et al. 2013; Mathew et al. 2014). Exposure to BPS disrupts cellular signalling in the apoptotic and survival pathways (Salvesen and Walsh 2014). Evidently, it is possible to expect the interference of BPS in signal pro-apoptotic pathways and signal cascades described also in gametes, leading to an altered cell cycle and cell death (Nevoral et al. 2013; Sedmikova et al. 2013). Further studies focused on the mechanism of BPS action are needed for a full understanding its negative effect on reproduction on the gamete level and cell cycle regulation.

In respect to previous regrettable substitution, another bisphenols, such as bisphenol F (BPF, bis(4-hydroxyphenyl)methane; see Figure 1), do

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not seem to be a suitable alternative. In addition to BPA and BPS, BPF has been described as endocrine disruptor as well (Perez et al. 1998). Surprisingly, natural presence of BPF has recently been observed in mustard and, therefore, it is a frequent compound of foodstuff (Zoller et al. 2016). Hence, BPF regulation is ambiguous for its chronical intake by a major part of human population (Dietrich and Hengstler 2016).

CONCLUSION

At present we are witnessing the substitution of BPA with BPS in a whole range of materials, and BPS is becoming a standard component of several products. BPS is a substance which is structurally very similar to BPA, it shows analogous effectiveness and mechanism of *in vitro* action. Biological changes occurring in the range of typical human exposures were documented at doses below those used in traditional toxicology. On the basis of the described comparisons, it is possible to expect that BPS, like BPA, is an endocrine disruptor, and that it may have similar targets and manner of action *in vivo* and may influence physiological processes on several levels. With regard to its slower degradation, BPS may act for a longer time in the organism and thus interfere with the regulation of reproduction of mammals in a yet more dangerous manner than has been demonstrated by a range of studies in the case of BPA.

The alarming results of the first reproduction studies on BPS have generated an acute need for a wider and at the same time more detailed assessment of the impacts of BPS, with emphasis on the area of reproduction of mammals, which is entirely lacking at present. Should this not materialize, due to the increasing industrial production of BPS caused by the need to replace BPA, unfortunately BPS may within the foreseeable future become just as great an environmental health risk as BPA. There is a need for very intensive research and subsequently also legislative measures in order to ensure that BPS will not become another "regrettable substitution" with pronounced negative impacts on the environment and on human health, including negative impacts on reproduction.

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Bisphenol S negatively affects the meotic maturation of pig oocytes

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

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

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

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

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

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

Results

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

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

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

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

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

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

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

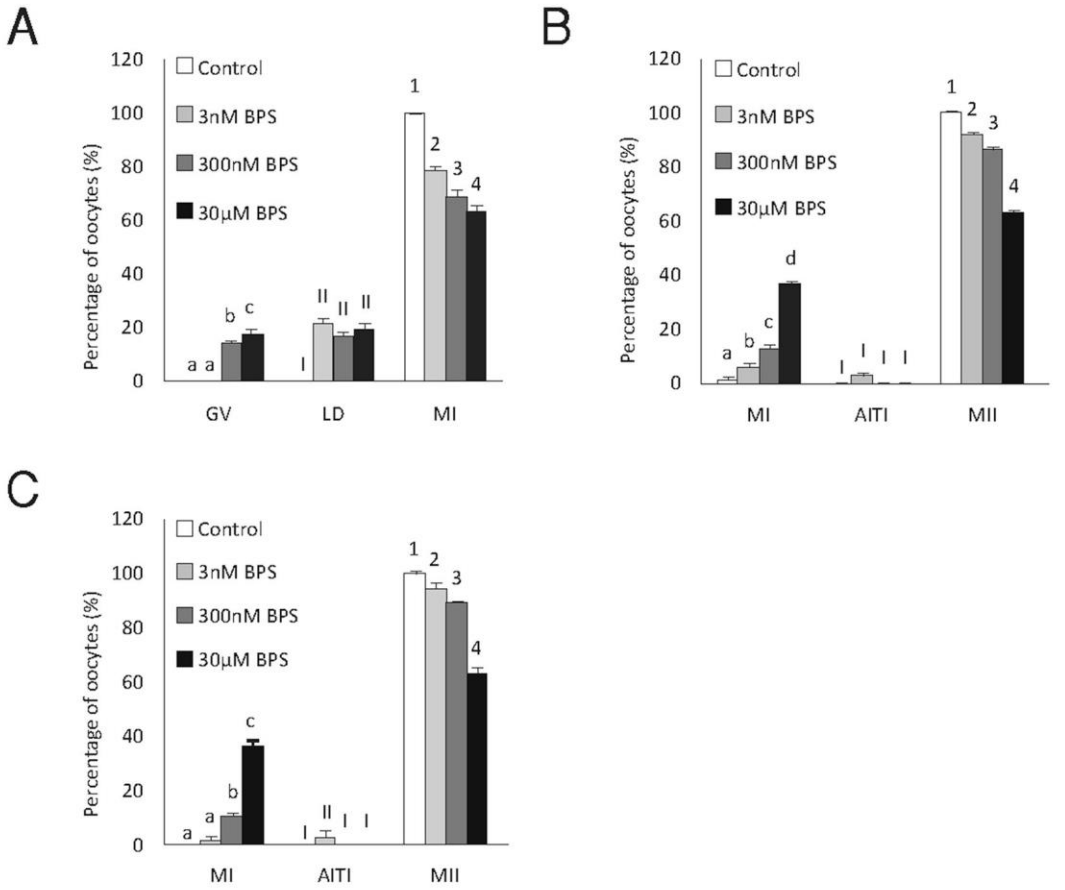

Figure 1. Effects of BPS on the meiotic maturation of oocytes. Effects of BPS (3 nM, 300 nM, and 30 μM) on the stages of meiotic maturation achieved by oocytes cultured for (**A**) 24 h, (**B**) 48 h, and (**C**) 72 h *in vitro*. GV – germinal vesicle, LD – late diakinesis, MI – metaphase I, AITI – anaphase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

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

Discussion

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

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

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

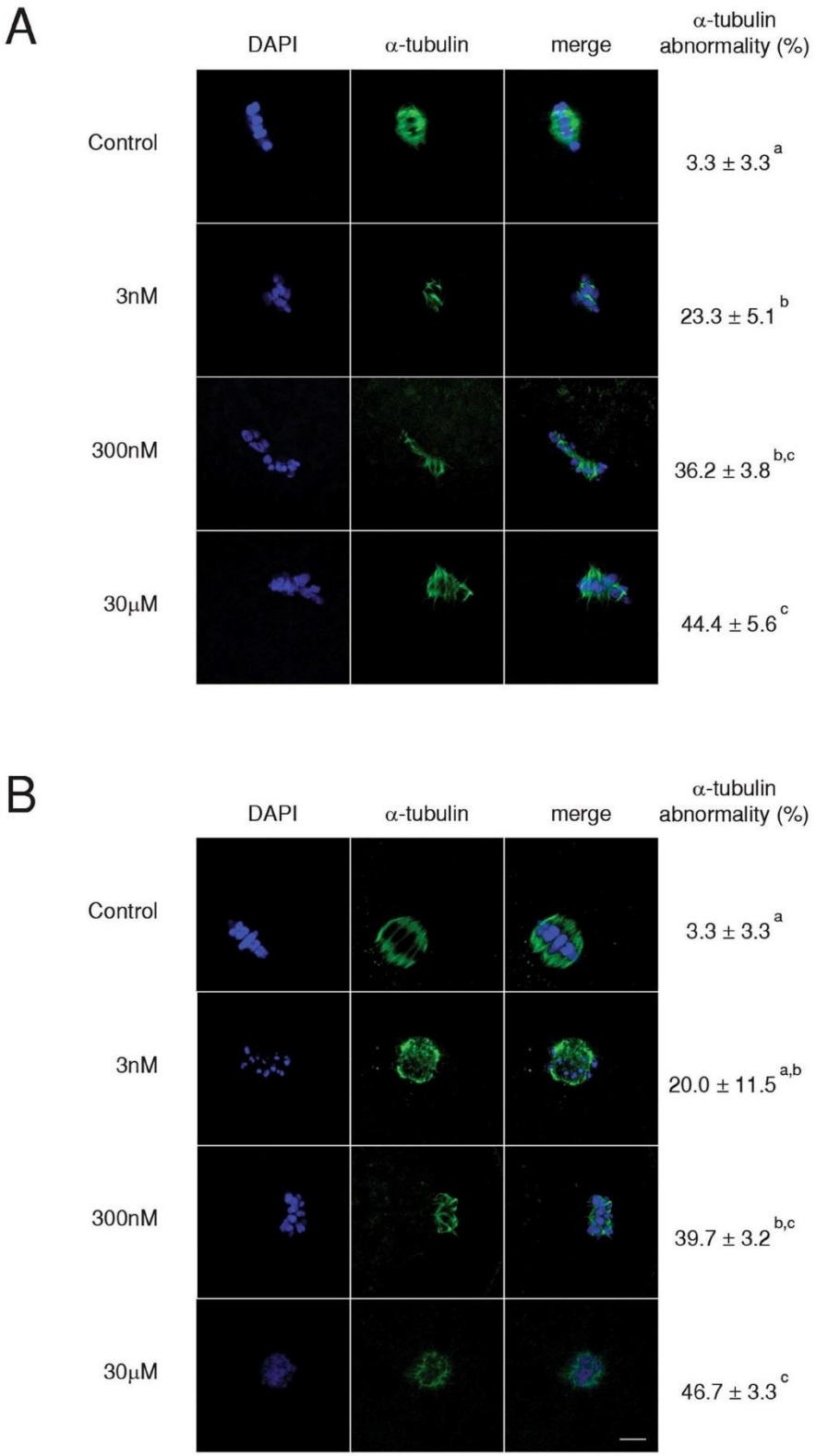

Figure 2. Effects of BPS on meiotic spindle formation during the maturation of porcine oocytes. Representative pictures showing defects in the morphology of spindle organization and chromosome alignment in oocytes after 24 h (**A**) or 48 h (**B**) of culture *in vitro* after BPS (3 nM, 300 nM, and 30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

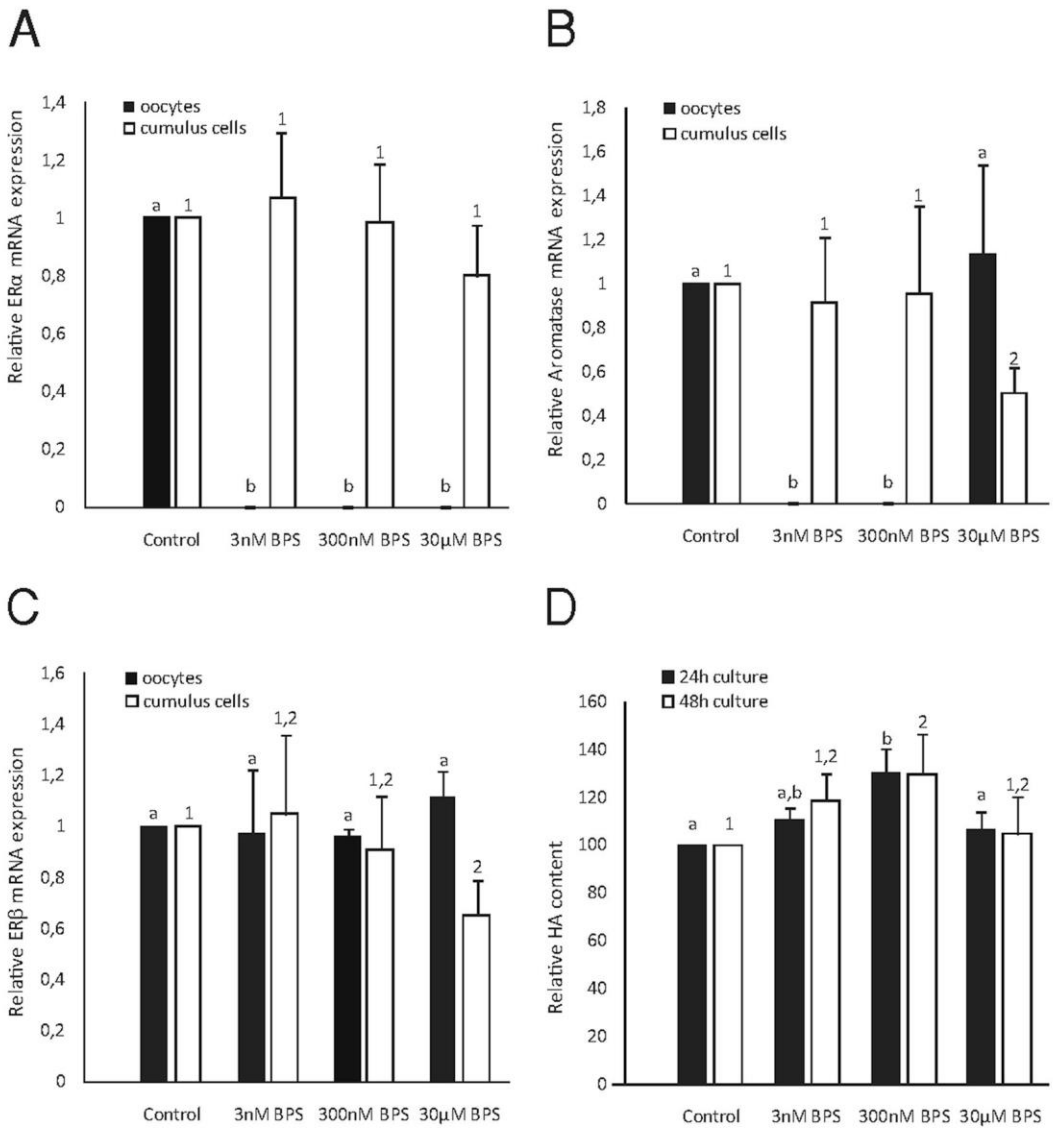

Figure 3. Effects of BPS on mRNA expression levels of selected genes and cumulus cell expansion. Effects of BPS (3 nM, 300 nM, and 30 μM) on the relative mRNA expression of (**A**) ERα, (**B**) ERβ, and (**C**) aromatase in oocytes and cumulus cells cultured for 48 h *in vitro*. The data are expressed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

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

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

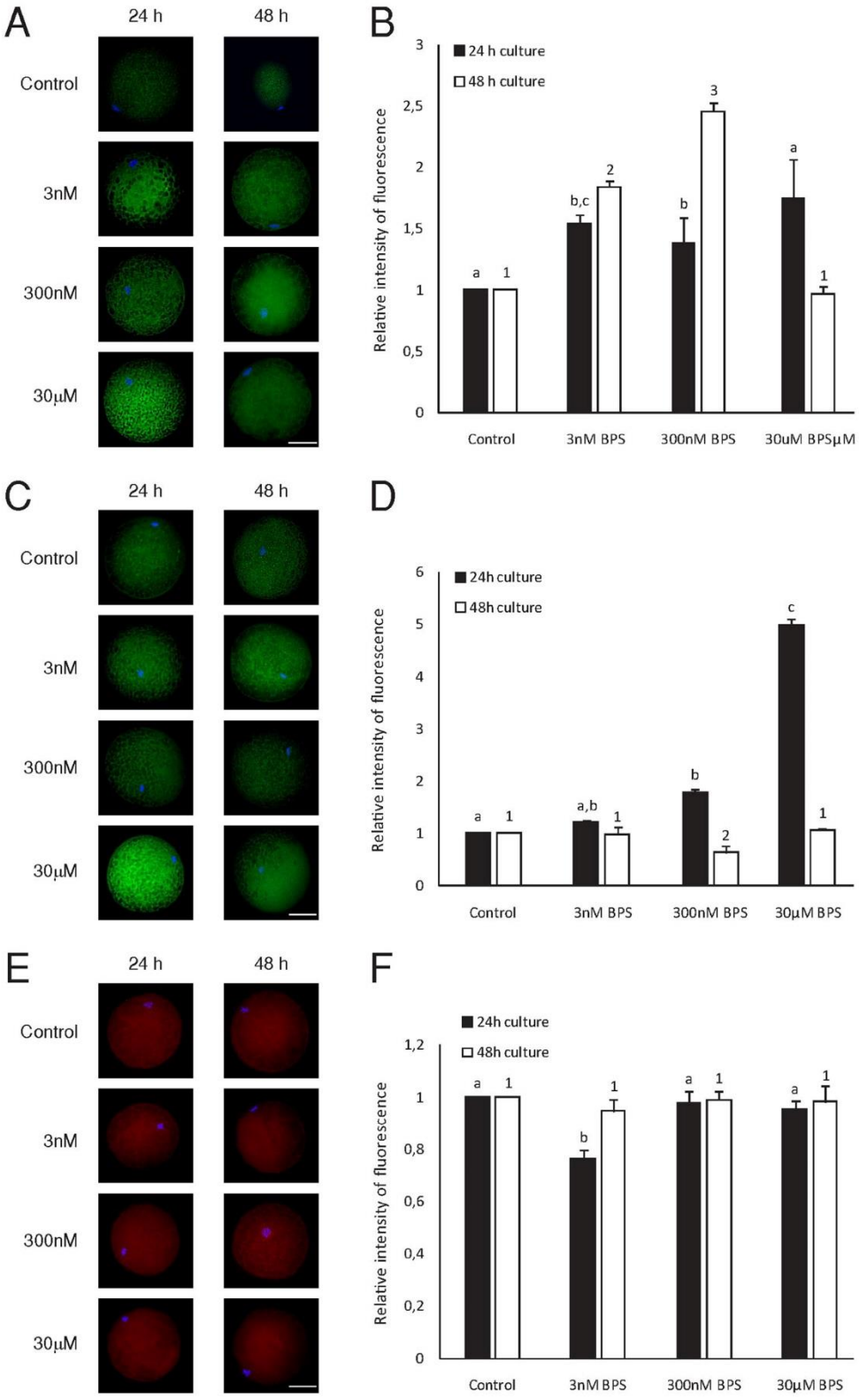

Figure 4. Effects of BPS on ERα, ERα, and aromatase during oocyte maturation. Representative pictures showing changes in the distribution of ERα (**A**), ERβ (**C**), and aromatase (**E**) in oocytes cultured for 24 h and 48 h *in vitro* after BPS (3 nM, 300 nM, and 30 μM) treatment. Green colour indicates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

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

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

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

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

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

Methods

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

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

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

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

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

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

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

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

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

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

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

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Author Contributions

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

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REPRODUCTION
RESEARCH

Long-term exposure to very low doses of bisphenol S affects female reproduction

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Abstract

Bisphenols belong to the endocrine disruptors, affecting reproduction even in extremely low doses. Bisphenol S (BPS) has become widely used as a substitute for the earlier-used bisphenol A; however, its harmlessness is questionable. The aim of this study was to evaluate the effect of BPS on folliculogenesis and oocyte quality after *in vivo* exposure to low doses of BPS. Four-week-old ICR females ($n = 16$ in each experimental group) were exposed to vehicle control (VC), BPS1 (0.001 ng BPS.g/bw/day), BPS2 (0.1 ng.g/bw/day), BPS3 (10 ng.g/bw/day) and BPS4 (100 ng.g/bw/day) for 4 weeks. Ovaries were subjected to stereology and nano liquid chromatography-mass spectrometry (LC/MS). Simultaneously, metaphase II oocytes were obtained after pregnant mare serum gonadotrophin and human chorionic gonadotrophin administration, followed by immunostaining. In particular, mating and two-cell embryo flushing were performed. We observed that BPS decreases the amount of ovarian follicles and BPS2 (0.1 ng.g/bw/day) affects the volume of antral follicles. Accordingly, ovarian proteome is affected after BPS2 treatment. While BPS2 dosing results mainly in cytoskeletal damage in matured oocytes, the effects of BPS3 and BPS4 seem to be due instead to epigenetic alterations in oocytes. Arguably, these changes lead to observed affection of *in vivo* fertilization rate after BPS3 and BPS4 treatment. BPS significantly affects female reproduction astoundingly in extremely low doses. These findings underline the necessity to assess the risk of ongoing BPS exposure for public health.

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Introduction

Bisphenol S (BPS) is an industrially produced compound found in many plastic items and, accordingly, a widespread environmental contaminant (Glausiusz 2014). BPS is found in water, house dust and/or food (Ike *et al.* 2006, Viñas *et al.* 2010, Liao *et al.* 2012*a*, Zhao *et al.* 2014, Li *et al.* 2015), entering the human body through ingestion, inhalation and dermal absorption (Liao *et al.* 2012*b*). Indeed, BPS has been detected in human blood and urine (Cobellis *et al.* 2009, Liao *et al.* 2012*a*, Yang *et al.* 2015). BPS is capable of disrupting the hormonal balance of the humans and influencing several physiological functions, similar to many other endocrine disruptors (EDs) (Colborn *et al.* 1993, Rochester & Bolden 2015). These chemical compounds mimic hormonal action in subtoxic doses and induce hormonal imbalance, leading to inadequate regulation of folliculo- and oogenesis (Mok-Lin *et al.* 2010, Vandenberg *et al.* 2012, Rivera *et al.* 2015). Recently, BPS has been used as a 'safe substitution' of mass-produced bisphenol A (BPA), previously determined to be a compound with ED features (Le Fol *et al.* 2017, Gingrich *et al.* 2018).

The negative effects of BPA on both male (Rahman *et al.* 2015, 2018) and female reproduction (Eichenlaub-Ritter *et al.* 2008, Rivera *et al.* 2015), on human reproductive health (Peretz *et al.* 2014, Mannelli *et al.* 2015), as well as on assisted reproductive technologies (ARTs) (Mok-Lin *et al.* 2010), have been described. Based on these alarming findings, the tolerable daily intake (TDI) for BPA (EFSA 2014, available from www.efsa.europa.eu) has been

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established at 50 ng.g/bw/day, without manifestation of toxic effects on live organisms. Recently, many plastic goods have a low BPA content, and some of them are labeled as BPA-free. However, BPA is gradually substituted by alternative compounds, most often structure analogues of BPA, including other bisphenols. Substantial evidence points to BPS as being the most used BPA alternative (Liao *et al.* 2012*c*, Barrett 2013, Eladak *et al.* 2015). An increasing number of studies (Rochester & Bolden 2015, Sartain & Hunt 2016, Žalmanová *et al.* 2017) underscore the possible deleterious substitutions known in other cases (Coggon 2002, Howard 2014).

A limited number of studies have focused on the biological mechanism of BPS and have indicated a BPA-like effect of BPS, with the ability of the artificial simulation of hormonal action, including interaction with estrogen receptors (Hashimoto *et al.* 2001, Chen *et al.* 2002, Kitamura *et al.* 2005, Kuruto-Niwa *et al.* 2005, Delfosse *et al.* 2012, Grignard *et al.* 2012, Rosenmai *et al.* 2014). According to those studies, genotoxicity, acute poisoning and/or inadequate estrogen activity associated with BPS were observed (Liao *et al.* 2012*a*). Moreover, in zebrafish offspring of exposed parents, there was also evidence of a BPS-induced decrease in the weight of gonads, disrupted production of steroidogenesis and increasing incidence of developmental malformations (Ji *et al.* 2013, Naderi *et al.* 2014). Regarding mouse sperm, a reduced quantity of spermatozoa and decreased sperm motility were observed after BPS *in vivo* treatment (Shi *et al.* 2017). The first evidence of the negative effect of BPS on mammalian oogenesis was recently published (Žalmanová *et al.* 2017), and damage to the meiotic spindle in mature oocytes treated *in vitro* has been described. In addition, BPS induces changes in the epigenetic pattern in somatic cells (Verbanck *et al.* 2017). A similar effect in gametes is possible.

To the best of our knowledge, there is a lack of study simulating temporal exposure of humans to BPS. Hence, this study addresses the impact of long-term *in vivo* dosing of mice with BPS in low concentrations, simulating real conditions of human exposure. In addition, experiments are designed with respect to the impact of BPS-exposed ART-subjected women. Our study simulates the exposure of the first and subsequent ovarian cycles until the reproductive peak is reached (~25 years in women, corresponding to 8-week-old mice), through hormonal ovarian stimulation followed by ART.

Materials and methods

Chemicals

All chemicals were purchased from Sigma-Aldrich if not otherwise stated.

Animals and ethical statements

All animal procedures were conducted in accordance with Act No. 246/1992 Coll., on the Protection of Animals against Cruelty, under the supervision of the Animal Welfare Advisory Committee at the Ministry of Education, Youth and Sports of the Czech Republic, approval ID MSMT-11925/2016-3. Four-week-old ICR mice were purchased from Velaz Ltd. (Prague, Czech Republic), housed in intact polysulfonate cages and maintained in a facility with a 12 h light:12 h darkness photoperiod, a temperature of 21 ± 1°C and a relative humidity of 60%. A phyto-estrogen-free diet 1814P (Altromin, AnimaLab, Poznan, Poland) and ultrapure water (in glass bottles, changed twice per week) were provided ad libitum.

Animal dosing and sample collection

Immediately after admission to the Animal Research and Care Facility, mice were randomly assigned to the experimental groups and allowed to acclimate for 1 week. BPS was then administered to the mice, and they underwent vaginal opening, for following 4 weeks through drinking water. The exposure consisted of five BPS dose treatments: 0, 0.004, 0.375, 37.5 and 375 ng/ml, that is vehicle control (VC) and BPS1–BPS4 groups, respectively. With respect to recorded body weight and known water intake (Bachmanov *et al.* 2002), the following doses were used: 0, 0.001, 0.1, 10 and 100 ng.g/bw/day (see 'Animal dosing' in the Supplementary methods for additional details; see section on supplementary data given at the end of this article). Appropriately comprehensive range of doses aimed to the endocrine-disrupting effect of BPS. Selected doses are in accordance with real oral exposure of human (Oh *et al.* 2018) and included the EFSA and NIH limits for the intake of BPA. The chosen window of exposure corresponds to at least three waves of cyclic follicle recruitment, beginning at the time of initial recruitment and continuing until attainment of the reproductive peak (McGee & Hsueh 2000, Moore-Ambriz *et al.* 2015).

Mice were euthanized by cervical dislocation and ovaries were collected. Body weight and weight of ovaries were recorded and ovaries were fixed as described in the following section. In addition to ovaries, blood was collected by intracardiac blood puncture. Serum samples were prepared by centrifugation (10,000 ***g***, 10 min, 4°C) and stored at −80°C until usage.

Ovarian histology

Ovaries free of oviduct and bursa were fixed in 2% paraformaldehyde for at least 48 h at room temperature. Histological analysis was performed as previously described (Hernandez-Ochoa *et al.* 2010) in accordance with the experimental design (Fig. 1). Seven left ovaries per each experimental group were used for histological and stereological analysis. Samples were dehydrated, embedded in paraffin blocks, cut into 10 µm-thick histological sections and mounted with five sections per slide. The histological cutting plane of each sample was randomized using an orientator (Mattfeldt *et al.* 1990). Every fifth slide was stained

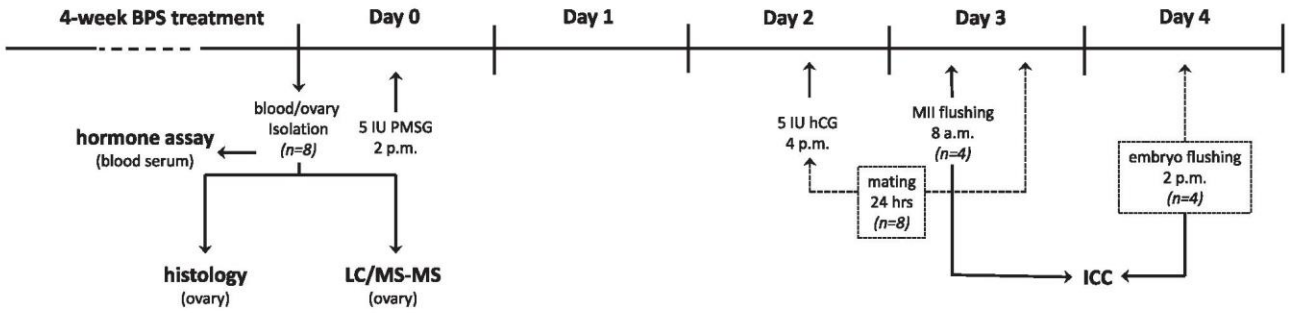


Figure 1 Experimental design. The scheme of hormonally unstimulated ovary collection for histological and proteomic analysis after 4-week bisphenol S (BPS) treatment, followed by evaluation of gonadotropin responsiveness expressed by flushing of *in vivo*–matured oocytes on Day 3. The following essential methodological approaches, such as ovarian histology, liquid chromatography-mass spectrometry (LC/MS-MS) and immunocytochemistry (ICC), are shown (blue lines). The number of mice per group used in a particular experiment is indicated.

in hematoxylin-eosin. From each stained slide, two adjacent sections, in predetermined order, were recorded. The primary, preantral, antral and atretic follicles and their volumes were morphologically assessed, with respect to analysis of recruited disruption-prone follicles, using a number of stereological techniques. For more details see 'Ovarian histology' in the Supplementary methods.

Proteomic analysis

Based on a pilot analysis covering all VC and BPS groups, ovaries of hormonally unstimulated mice ($n=3$) of VC and BPS2 groups were collected for the complete proteomic analysis. Nano liquid chromatography-mass spectrometry (LC/MS) was used for protein identification and quantification. For additional details, see 'Protein identification by nano LC/MS' in the Supplementary methods.

Assessment of ovarian gonadotropin responsiveness

Following pregnant mare serum gonadotrophin (PMSG)/human chorionic gonadotrophin (hCG) treatment of females (treated as illustrated in Fig. 1), flushing of *in vivo*–matured oocytes was performed. The total number and percentage of vital matured oocytes were counted and these oocytes were used for immunocytochemistry.

In vivo *fertilization assay*

Following PMSG/hCG treatment of females as described earlier, an *in vivo* fertilization assay was performed according to Zudova *et al.* (2004). Embryos were flushed from the oviduct and the fertilization rate was calculated as the percentage of cleaved embryos of the total recovered embryos/oocytes. For more details, see Fig. 1 (Experimental design).

Immunocytochemistry (ICC)

Oocytes were fixed and permeabilized using two different methods according to visualized factors (for more details, see 'Immunocytochemistry' in the Supplementary methods). After blocking in 1% BSA in PBS with Tween 20, incubation with specific antibodies followed: anti-α-tubulin (Sigma-Aldrich), pericentrin (PCNT; Abcam), anti H3K27me2 (H3K27me2; Abcam) and anti-5′-methyl cytosine (5meC; Abcam). Thereafter, samples were washed and incubated with a cocktail of anti-mouse Alexa Fluor 488 and anti-rabbit Alexa Fluor 647. Concurrent with washing, phalloidin was applied for β-actin visualization. The signal intensity (i.e. integrated density) was measured using ImageJ software (NIH).

Statistical analysis

The data were processed with Statistica Cz 12 (StatSoft, Inc., Tulsa, OK, USA). For overall comparison of the study groups, Kruskal–Wallis ANOVA (for quantitative variables) or chi-square tests (for proportions) were used. In the case of a significant overall finding, differences between individual group pairs were assessed *post hoc*, using multiple comparisons of mean ranks, the Mann–Whitney *U* test with Bonferroni correction, or Fisher's exact test with Bonferroni correction. Where appropriate, correlations among variables were assessed using Spearman's method. The level of statistical significance was set at $\alpha=0.05$ and all reported *P* values and tests were calculated as two-tailed.

Results

Effect of BPS on ovarian follicles and histological quality

The goal of this experiment was to test the effect of BPS on the ovaries of hormonally unstimulated outbred mice. The BPS treatment covered the developmental time period, which includes initial follicle recruitment and subsequent cyclic ovarian recruitments, until the reproductive peak was reached, as evidenced by tracking of all recruited follicle stages (i.e. primary, preantral and antral follicles). The primary quantitative data, with the results of statistical and correlational analysis, are provided in the Supplementary results (Supplementary Table 2).

A significant reduction in the total volume of the ovary and relative ovary weight was found (Fig. 2A and B). This can largely be attributed to the reduction of the corpus luteum volume, as confirmed by the correlational

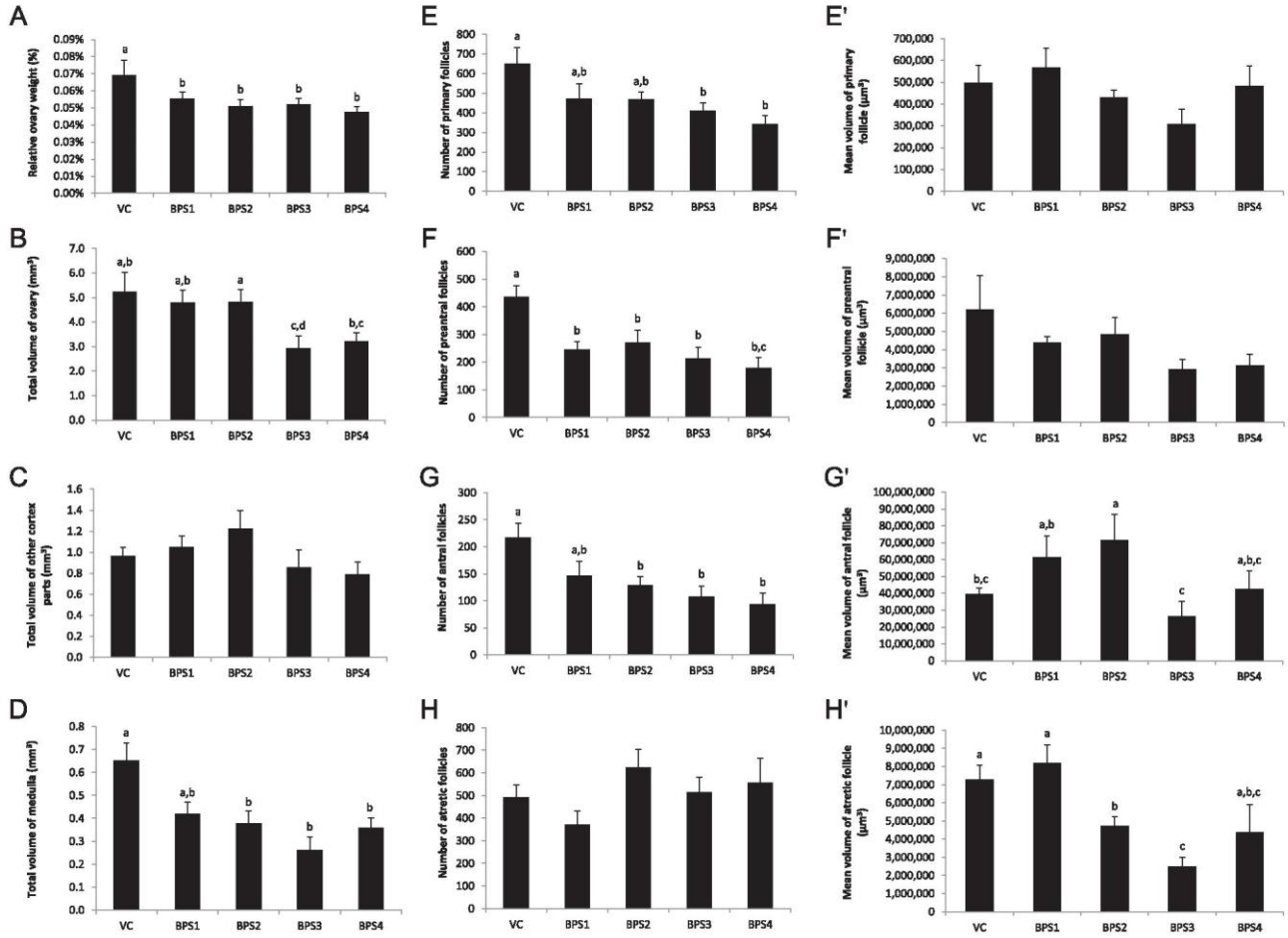


Figure 2 Ovarian histology after treatment with different doses of BPS. The weight of ovaries related to total body weight (A) and volume of ovaries (B), including ovarian cortex (C) and medulla (D), were recorded. Different superscripts indicate statistically significant differences ($P \leq 0.05$). Absolute numbers (E, F, G and H) and indicate volumes (E′, F′, G′ and H′) of primary, preantral, antral and atretic follicles are evidenced per one (left) ovary. Data are expressed as the mean ± SEM. Different superscripts indicate statistically significant differences ($P \leq 0.05$).

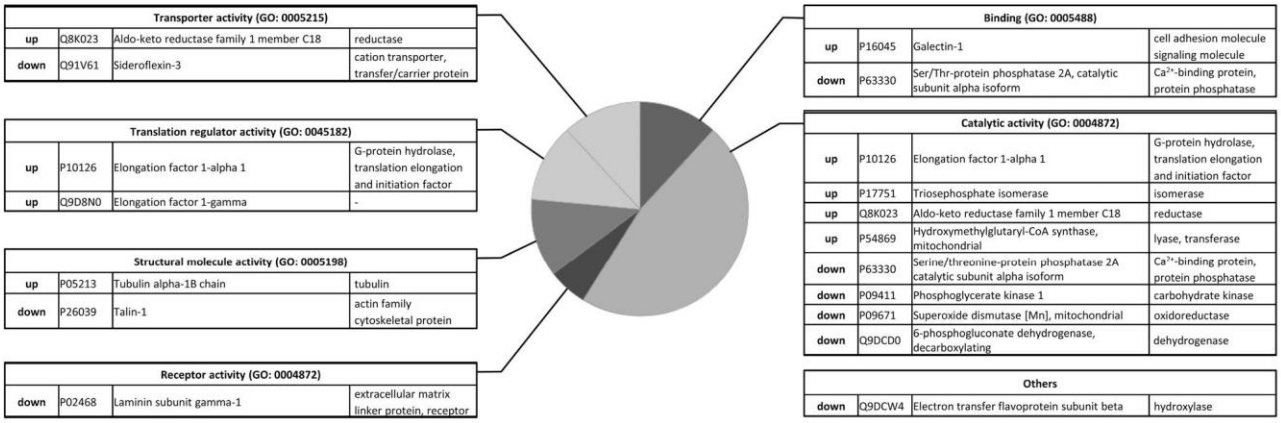
assessment (Supplementary Table 2 of Supplementary results), as well as to the decrease of both the number of antral follicles and their volume (Fig. 2G and G′). In addition to the cortex structures, medulla volumes were also reduced after BPS treatment (Fig. 2D).

No significant differences were found among the mean volumes of primary or preantral follicles in the administered BPS concentrations (Fig. 2E′ and 2F′). In contrast, the mean volume of antral follicles was increased almost twofold in animals receiving BPS in low concentrations (Fig. 2G′). While the number of primary and preantral follicles followed a linear dose-dependent curve (Fig. 2E and F), the volume of the antral and atretic follicles shows a non-linear effect (Fig. 2G′ and 2H′). Moreover, in the BPS3- and BPS4-treated animals, a visible antrum appears in follicles with fewer layers of granulosa cells (Supplementary Fig. 1). These findings were reflected by a significant decrease in plasma 17β-estradiol after BPS3 and BPS4 treatment (Supplementary Fig. 2).

Proteomic profiling of the ovary after BPS treatment

This experiment was focused on possible protein targets of BPS, and an LC/MS-MS analysis was performed on hormonally unstimulated mouse ovaries. Based on proteomic analysis of BPS-wide exposure, BPS2 treatment was selected for additional detailed screening. There were 171 identified and quantified proteins, varying in densitometry analysis between VC and BPS2.

Apparent up-/or downregulated protein targets were identified, belonging to proteins with catalytic activity (e.g., superoxide dismutase (SOD)), transporter activity (e.g., aldo-keto reductase), translation regulator activity (elongation factors), structure molecule activity (e.g., tubulin α-1B chain, talin), receptor activity (laminin subunit), basal metabolism (subunit β of electron transfer flavoprotein) and binding proteins (galectin, Ca^{2+}-binding Ser/Thr-protein phosphatase 2A). Proteins identified as affected by the BPS2 treatment are



Transporter activity (GO: 0005215)			
up	Q8K023	Aldo-keto reductase family 1 member C18	reductase
down	Q91V61	Sideroflexin-3	cation transporter, transfer/carrier protein

Translation regulator activity (GO: 0045182)			
up	P10126	Elongation factor 1-alpha 1	G-protein hydrolase, translation elongation and initiation factor
up	Q9D8N0	Elongation factor 1-gamma	-

Structural molecule activity (GO: 0005198)			
up	P05213	Tubulin alpha-1B chain	tubulin
down	P26039	Talin-1	actin family cytoskeletal protein

Receptor activity (GO: 0004872)			
down	P02468	Laminin subunit gamma-1	extracellular matrix linker protein, receptor

Binding (GO: 0005488)			
up	P16045	Galectin-1	cell adhesion molecule signaling molecule
down	P63330	Ser/Thr-protein phosphatase 2A, catalytic subunit alpha isoform	Ca^{2+}-binding protein, protein phosphatase

Catalytic activity (GO: 0004872)			
up	P10126	Elongation factor 1-alpha 1	G-protein hydrolase, translation elongation and initiation factor
up	P17751	Triosephosphate isomerase	isomerase
up	Q8K023	Aldo-keto reductase family 1 member C18	reductase
up	P54869	Hydroxymethylglutaryl-CoA synthase, mitochondrial	lyase, transferase
down	P63330	Serine/threonine-protein phosphatase 2A catalytic subunit alpha isoform	Ca^{2+}-binding protein, protein phosphatase
down	P09411	Phosphoglycerate kinase 1	carbohydrate kinase
down	P09671	Superoxide dismutase [Mn], mitochondrial	oxidoreductase
down	Q9DCD0	6-phosphogluconate dehydrogenase, decarboxylating	dehydrogenase

Others			
down	Q9DCW4	Electron transfer flavoprotein subunit beta	hydroxylase

Figure 3 Quantification analysis of ovarian proteins after BPS2 treatment. Proteins with significantly different levels ($P \leq 0.05$) in the BPS2 group, compared to vehicle control (VC), are shown and summarized. Nano LC/MS protein detection was performed, followed by analysis using ProteinPilot software and interpretation with the use of Panther 12.0 software. There are 15 protein hits, representing consistent upregulated (up) or downregulated (down) trends, at least in two individual samples per analyzed experimental group (i.e. VC and BPS2; $n = 3$ each, from independent experiments). Proteins include binding proteins and proteins with different activities (transporter, translation regulator, structural, receptor and catalytic).

summarized in Fig. 3 (also see the separately provided Supplementary Tables 1 and 2 for complete output).

Effect of BPS on chromosome misalignment and spindle malformation in oocytes

The goal of this experiment was to evaluate the influence of BPS treatment on spindle formation and chromosome segregation in *in vivo*–matured and flushed oocytes. In addition to spindle α-tubulin and chromatin staining, subcellular immunolocalization of PCNT was performed.

An increased incidence of spindle malformation and abnormal chromosome alignment was observed in BPS-treated groups (Fig. 4A). After BPS2 and BPS3 treatments, there were increased numbers of abnormal oocytes, including those with spindle- and/or chromatin-derived oocyte abnormalities, (30.6 ± 5.9 and 34.3 ± 3.2%, respectively, vs. 15.0 ± 3.4% in VC). These deviations seem to have been caused by spindle malformation, for which statistically significant differences were observed after BPS2 and BPS4 treatment (27.8 ± 10.7 and 33.7 ± 5.5%, respectively, vs. 9.0 ± 2.6%). To the contrary, there were no significant differences in chromosome misalignments among the groups. Data are summarized in Fig. 4B.

The observation of malformed spindle α-tubulin was supported by the results of the PCNT staining (see the representative pictures in Fig. 4C). Indeed, a PCNT signal was observed seemingly external to the spindle pole in the BPS1 group (dashed arrowhead in Fig. 4C), while oocyte spindle abnormalities in the BPS2 group were accompanied by a cap-shaped pole (empty arrowhead). Similarly, divided PCNT poles in the matured oocytes in metaphase II (MII) oocytes were found in greatest abundance in BPS4-treated mice (white arrowhead).

Effect of BPS on oocyte yield, fertilization ability and epigenetic quality of oocytes

The goal of this experiment was to examine the physiological function of BPS-influenced ovaries of mice. PMSG-hCG hormonal stimulation was used and efficiency of *in vivo*–matured oocytes in MII stage was evaluated. Subsequently, genome-wide 5-methylated cytosine (5meC) and di-methylated histone H3 on lysine K27 (H3K27me2) were analyzed, as the DNA and histone markers of heterochromatin establishment and chromatin stability. In addition, an *in vivo* fertilization assay was performed as a marker of the fertilization ability of oocytes.

There was an increased number of MII oocytes flushed after hormonal stimulation following BPS1, BPS3 and BPS4 treatments; however, these differences were not statistically significant (Fig. 5A). The 5meC and H3K27me2 were immunolocalized in MII oocytes thereafter, and the signal intensity was related to the control oocytes (Fig. 5B). Although 5meC was not affected after BPS treatment compared with the VC group, differences among individual BPS treatment groups were observed (Fig. 5C). In contrast to 5meC, the methylation of H3K27 was affected after BPS3 and BPS4 treatment (Fig. 5D). In addition to oocyte epigenetic quality, the fertilization rate (fertilized oocyte/two-cell embryo ratio) was affected after BPS exposure. Decreasing fertilization rate in dose-dependent manner is intimated; however, only BPS3 showed statistically significant decline of 19.2%. On the contrary, BPS4 significantly increased the fertilization rate. The number of two-cell embryos

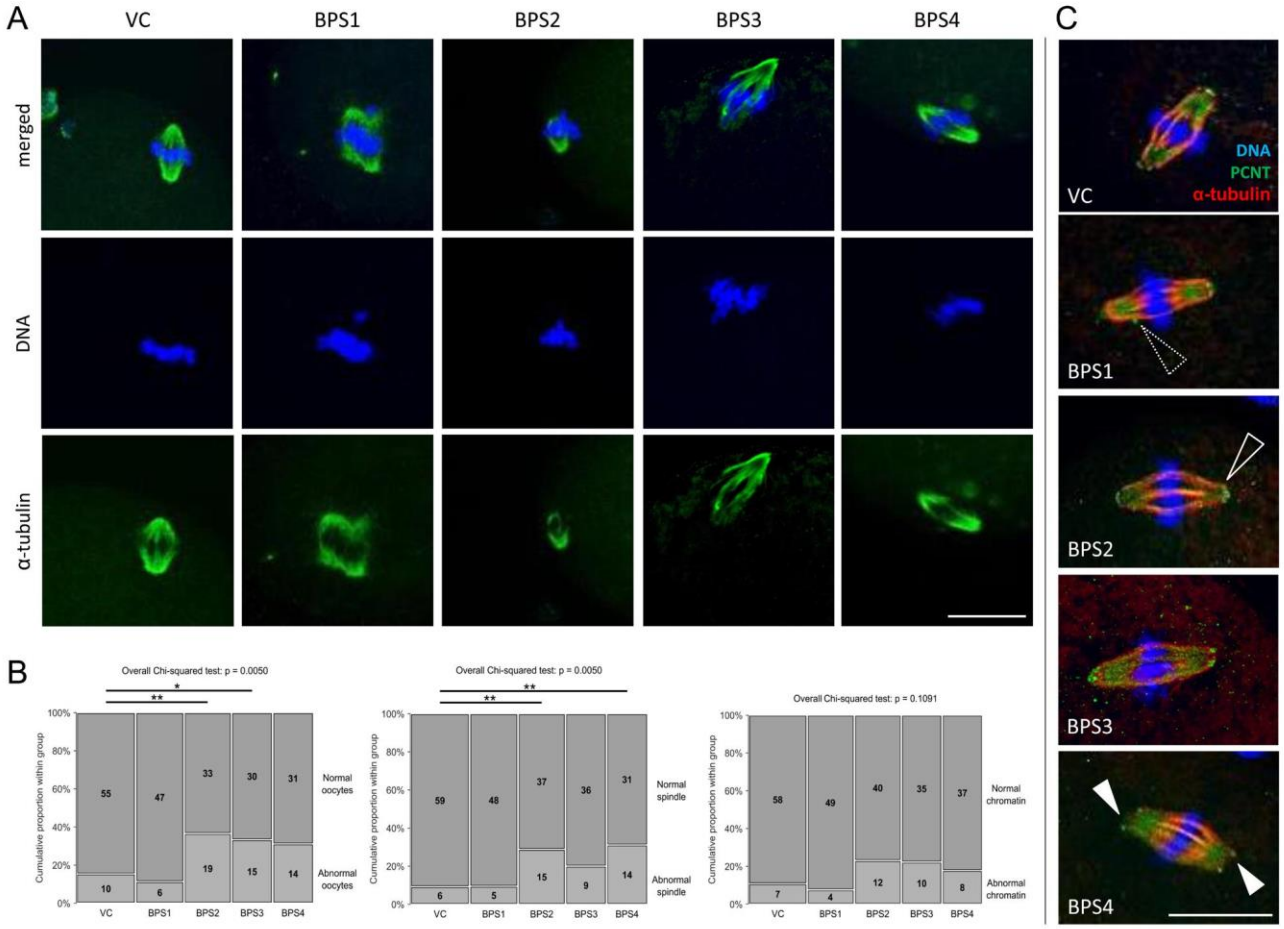


Figure 4 Incidence of spindle misalignment and abnormal chromosome segregation of *in vivo*–produced oocytes following bisphenol S (BPS) treatment. Abnormal oocytes, including abnormal spindle and/or chromatin in metaphase plate (A), were counted and quantified (B). Groups BPS1–BPS4 were tested against the vehicle control (VC) group for significant differences in the proportion of abnormal findings, that is abnormal oocytes in general, abnormal spindle and abnormal chromatin alignment. Fisher's exact test was used; one star indicates significance at the basic level ($P<0.05$), two stars indicate significance after Bonferroni correction for quadruple testing ($P<0.0125$) and non-significant results are not shown. Observed numbers of oocytes with normal and abnormal phenotype are stated inside the appropriate columns. The width of the columns is proportional to the number of oocytes analyzed in each group (number of flushed females = 3 per each experimental group). In addition to spindle α-tubulin, pericentrin (PCNT) on the poles of the spindle was tracked. Arrowheads indicate abnormal formation of PCNT on spindle poles (C). Scale bars represent 25 µm.

flushed per mouse did not show significant differences (Fig. 5E).

Discussion

Our study provides the first evidence of the effect of BPS on mammalian general ovarian morphology and physiology, including folliculogenesis, responsiveness to hormonal stimulation and the quality of *in vivo*–matured oocytes. Tested doses (0.001–100 ng.g/bw/day) with observed BPS effects are very low, comparable to established values for the TDI (50 ng.g/bw/day), no-observed-adverse-effect level (5 µg.g/bw/day) or low-observed-adverse-effect level (50 µg.g/bw/day) for BPA (Tyl 2009). Moreover, there was no possibility of strictly testing BPS with regard to these values, because they have not yet been established. Tested very low doses of BPS represent real environmental exposure levels of people (Liao *et al.* 2012*a*,*b*, Rocha *et al.* 2015) and deserve rigorous attention.

We simulated the heterogeneity in the human population by using hormonally unstimulated mouse females. Although mice were in various stages of the estrus cycle, we observed a significant reduction in the total number of activated primary and preantral follicles after animals were exposed to various doses of BPS. The decrease in the number of follicles was accompanied by an increased volume of antral follicles, which could be a compensatory reaction, as the follicle population is regulated by follicular interactions, perhaps by a quorum sensing mechanism (Bristol-Gould *et al.* 2006). This compensation seems to have its limits, because

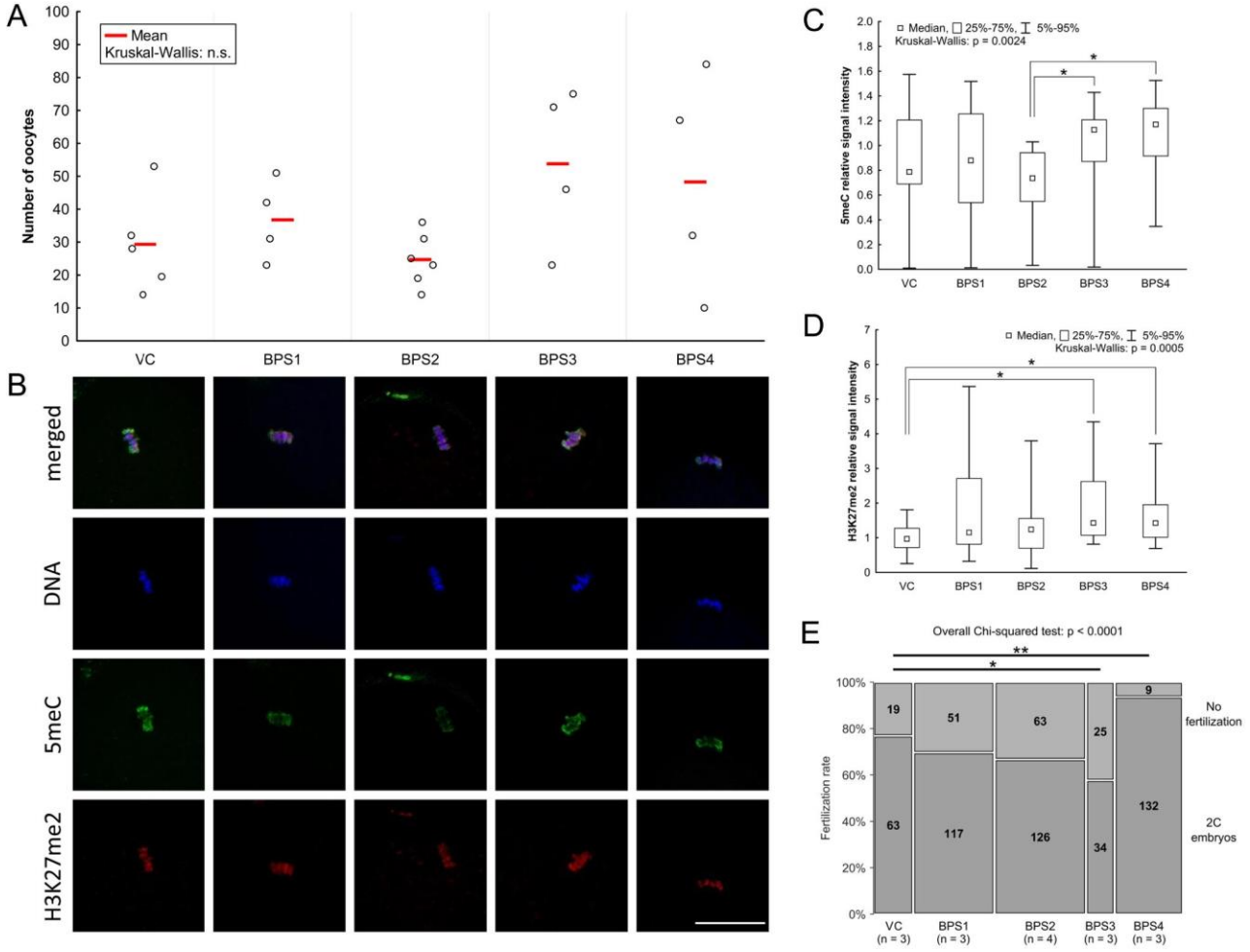


Figure 5 The effect of bisphenol S (BPS) on *in vivo* oocyte production, oocyte fertilization ability and epigenetic quality. Efficiency of *in vivo* flushing of matured oocytes (number of matured matured oocytes in metaphase II (MII) oocytes per one mouse) after BPS treatment, followed by gonadotropin stimulation (A). Immunostaining (B) and relative signal intensity (vehicle control (VC) = 1) of 5-methylcytosine (5meC) (C) and H3K27me2 (D) in *in vivo*–matured oocytes. 5meC and H3K27me2 data are expressed as means. The boxplot shows 25% and 75% quantiles, as well as 5% and 95% quantiles of observed it in the groups. One star indicates significance at the basic $P \leq 0.05$ level (C and D). Scale bar represents 25 µm. Fertilization rate of ovulated oocytes was recorded after *in vivo* fertilization assay (E). Groups BPS1–BPS4 were tested against the VC group for significant differences in the proportion of fertilized oocytes (fertilization rate) using Fisher's exact test (one star indicates significance at the basic level $P < 0.05$, two stars indicate significance following Bonferroni correction for quadruple testing, $P < 0.0125$, non-significant results are not shown). Numbers of non-fertilized oocytes (no fertilization) and 2C embryos are stated inside the appropriate columns. The width of the columns is proportional to the number of flushed oocytes/2C embryos for the particular group. Numbers of flushed females are indicated in brackets. There is no difference in the number of flushed two-cell (2C) embryos per mouse.

at higher doses of BPS, we observed a decreased volume of antral follicles, which could be associated with suppression of their development (Altunkaynak *et al.* 2016). These findings are supported by hormone profiling and the observed decrease of β17-estradiol in blood serum (Supplementary results). Although BPS is considered to be an analogue of BPA, the effects of the two bisphenols could differ in many aspects, as Moore-Ambriz *et al.* (2015) did not observe effects of BPA on histological features of ovaries after comparable dosing of mice. Contrary to our findings, which did not reveal an increase in follicle atresia after BPS treatment, Soleimani Mehranjani and Mansoori (2016) observed an increased number of atretic follicles after treatment of mice with BPA.

Because the effect of BPS on ovaries and folliculogenesis was observed even at a low dose (0.1 ng.g/bw/day), the proteome of hormonally unstimulated ovaries after BPS2 treatment followed. The decreasing level of SOD suggests a limited ability to be resistant to oxidative stress, similar to the previously described suppression of SOD expression and activity after treatment of bisphenols (Maćczak *et al.* 2017, Rahman *et al.* 2017). This assumption is further supported by our observation of the affected expression of proteins associated with basal metabolism

(aldo-keto reductase, hydroxymethylglutaryl-CoA synthase and phosphoglycerate kinase) and protein-glutamine γ-glutamyltransferase, pro-apoptotic and inflammatory markers. In addition to cellular stress, based on BPS-wide proteomic screening, the factors belonging to steroidogenesis, such as 7-dehydrocholesterol reductase, apolipoprotein and lipid transfer protein, were altered. In particular, 7-dehydrocholesterol, involved in steroid metabolism (Shackleton 2012), was significantly decreased after BPS1, BPS2 and BPS3 treatment. This effect can be considered as being a feature of endocrine disruption, with respect to the significant decrease of circulating 17β-estradiol, mentioned earlier. This assumption is in accordance with BPA-disrupted steroidogenesis and estradiol biosynthesis (Peretz *et al.* 2011), as well as the observation of BPA-comparable estrogenic activity of BPS (Grignard *et al.* 2012). In addition, BPS treatment decreased the amount of protein-arginine N-methyltransferase 1, and post-translational modifications of various proteins, including factors essential for fertilization and early embryonic development (Panamarova *et al.* 2016), are considerably affected. This observation confirms the likelihood of BPS-mediated disruption of epigenetic modulation. In addition to steroidogenesis and epigenetics, cytoskeletal and associated factors (tubulin α-1B chain, talin-1, Ser/Thr-protein phosphatase 2A) are affected and considerably affiliated with other findings on cytoskeletal structures, accordingly with previous studies on BPA (Rahman *et al.* 2016).

Although the follicle populations were mostly reduced after BPS treatment and proteomics were significantly affected, the responsiveness on gonadotropin stimulation expressed by the number of flushed *in vivo*–matured oocytes was unchanged. Similar to oocyte number, the number of flushed two-cell embryos did not show a significant change in mice treated with BPS. This could indicate that superovulatory treatment still permits recruitment of a satisfactory number of follicles from a reduced follicular population. However, in humans, there is a reduced response to ovarian stimulation in women with reduced population of antral follicles in ovaries (Hendriks *et al.* 2005). The quality of flushed embryos was not determined in our study. Because we did not observe fragmented embryos, it does not appear that apoptosis poses a problem in these very early stages of embryonic development (Kamjoo *et al.* 2002, Tarín *et al.* 2002). On the other hand, we can suggest impaired quality of embryos with later manifestations as a result of detrimental BPS effect on oocytes (Krisher 2004, Sirard *et al.* 2006). Further experiments are needed to follow the effects of BPS exposure on embryo quality.

An epigenetic effect of EDs is one of their well-defined molecular mechanisms. Accordingly, we analyzed DNA and histone methylation, tracking 5meC and dimethylation of lysine K27 on histone H3 (H3K27me2), respectively. Changes in signal intensity of H3K27me2 were observed after BPS3 and BPS4 treatment, demonstrating an epigenetic mode of BPS action. Our observations are in accordance with previous studies describing BPA-affected methylation of core histones H3 and H4 in oocytes (Trapphoff *et al.* 2013, Wang *et al.* 2016). This suggests that BPS-modulated epigenetically derived gene imprinting occurs, as previously demonstrated for BPA (Trapphoff *et al.* 2013). In general, although the quantity of flushed oocytes and embryos are not affected, the quality of the cells can be influenced with numerous consequences in subsequent processes, such as fertilization, embryonic development and/or gametogenesis of offspring where transgenerational inheritance can occur (Dolinoy *et al.* 2007, Ziv-Gal *et al.* 2015). This assumption is supported by the aforementioned BPS3/BPS4-shifted fertilization rate.

Observed aberrant spindles of BPS-derived *in vivo*–matured oocytes are in accordance with the previously described negative effect of BPA on the meiotic spindle of mammalian oocytes (Hunt *et al.* 2003, Can *et al.* 2005, Viñas *et al.* 2010, Wang *et al.* 2016, Zhang *et al.* 2017). So far, the BPS destructive effect on oocyte spindles has been described in vitro (Žalmanová *et al.* 2017). In addition to spindle α-tubulin, PCNT was tracked and its disruption was detected. In parallel with our observation, abnormal BPA-induced localization of PCNT has been described (Eichenlaub-Ritter *et al.* 2008). However, the molecular mechanism remains unknown and, based on differences in the BPS-dose-dependent pattern of PCNT localization, the BPS effect seems to be dose-specific. In accordance with a previous study (Jiao *et al.* 2017), it is likely that there are BPS-targeted proteins belonging to the cytoskeleton and to up-stream factors leading to cytoskeleton formation. In addition to the effects on cytoskeletal proteins, abnormalities in chromosome segregation were observed and the mechanism of chromosome alignment is presumably affected, similarly to previous BPA studies (Hunt *et al.* 2003). Nevertheless, further experiments focused on the assessment of chromosome alignment are needed.

As a group, higher BPS concentrations (BPS3 and BPS4, i.e. 10 and 100 ng.g/bw/day) seem to be targeting steroidogenesis. In addition to this hormonal effect, BPS3/4-modulated epigenetics is clear, and the changes in hormonal signalization, epigenetic changes and fertilization capability are clearly connected. However, the ambivalence of the BPS effect on fertilization capability notes a difference in the molecular mechanism, even between BPS3 and BPS4. In contrast to BPS3 and BPS4, the dose of BPS2 (0.1 ng.g/bw/day) led to cytoskeletal damage with clear manifestations. The further impact of different forms of BPS-damaged oocytes on embryonic development remains to be elucidated.

Conclusions

In conclusion, this study is the first thorough evaluation of female reproduction following *in vivo* BPS treatment. In comparison with BPS-substituted BPA, BPS shows comparable biological effects even at much lower doses. Moreover, BPS is chemically more stable, making it worse in terms of biodegradability than BPA, and it shows higher levels of dermal penetration than BPA (Ike *et al.* 2006, Danzl *et al.* 2009, Liao *et al.* 2012*a*). Therefore, our conclusions indicate the necessity to estimate BPS intake in human populations and to assess the risk of ongoing BPS exposure to public health.

Supplementary data

This is linked to the online version of the paper at https://doi.org/10.1530/REP-18-0092.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Epigenetic and non-epigenetic mode of SIRT1 action during oocyte meiosis progression

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Abstract

Background: SIRT1 histone deacetylase acts on many epigenetic and non-epigenetic targets. It is thought that SIRT1 is involved in oocyte maturation; therefore, the importance of the ooplasmic SIRT1 pool for the further fate of mature oocytes has been strongly suggested. We hypothesised that SIRT1 plays the role of a signalling molecule in mature oocytes through selected epigenetic and non-epigenetic regulation.

Results: We observed SIRT1 re-localisation in mature oocytes and its association with spindle microtubules. In mature oocytes, SIRT1 distribution shows a spindle-like pattern, and spindle-specific SIRT1 action decreases α-tubulin acetylation. Based on the observation of the histone code in immature and mature oocytes, we suggest that SIRT1 is mostly predestined for an epigenetic mode of action in the germinal vesicles (GVs) of immature oocytes. Accordingly, BML-278-driven trimethylation of lysine K9 in histone H3 in mature oocytes is considered to be a result of GV epigenetic transformation.

Conclusions: Taken together, our observations point out the dual spatiotemporal SIRT1 action in oocytes, which can be readily switched from the epigenetic to non-epigenetic mode of action depending on the progress of meiosis.

Keywords: Epigenetics, Histone code, *In vitro* maturation, Oocyte, SIRT1, Sirtuin 1

Background

SIRT1, a mammalian homologue of yeast Sir2, belongs to NAD^+-dependent histone deacetylases (also called sirtuins, SIRT1–7) [1]. SIRT1 shows the ability to deacetylate both epigenetic and non-epigenetic targets; therefore, SIRT1 molecular action leads to regulation of the cell cycle, apoptosis, and oxidative stress response, thereby influencing cell viability and senescence [2–4]. Resveratrol and several other polyphenolic compounds have been identified as sirtuin-activating, and their positive effect on oocyte viability was due to sirtuin activation [5–7]. However, an exact mechanism of SIRT1 action in oocytes has not been studied and, therefore, practical use of SIRT1-stimulating compounds remains to be limited.

Mammalian oocytes represent a unique model for the study of cell cycle regulation. Oocyte meiosis is synchronised in the G2/prophase and arrested for several years or decades in domestic animals and humans, respectively. Meiosis re-initiation, accompanied by nuclear envelope breakdown (NEBD) of germinal vesicles, is followed by further meiotic progression and finally, a mature oocyte is arrested again in metaphase of the second meiotic round (metaphase II), where the oocyte is predestined for fertilisation. The oocyte meiotic maturation is ingeniously orchestrated by the machinery of enzymes responsible for post-translational modifications (PTMs) of protein targets (summarised by Madgwick and Jones [8]). In addition to the well-known protein

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phosphorylation balanced by phosphatases/kinases [9–12], sirtuins represent an impactful protein-modulating acetylation of many cytoskeletal and/or regulatory proteins [13–16]. Moreover, SIRT1-driven deacetylation is involved in epigenome establishment, and SIRT1 represents an epigenetic factor affecting male germ cells [17] as well as early embryos [18]. Accordingly, SIRT1 involvement in oocyte epigenome modulation is taken into consideration.

There are many direct epigenetic SIRT1 substrates, i.e., histone lysine (K) residues, such as H3K9, H4K16 and others [19–21]. In a previous study, we revealed a SIRT1-modified histone code favouring histone H3K9 methylation in one-cell zygote [18]. This histone modification represents a relevant marker of SIRT1 activity through the associated signalling of SUV39H1 methyltransferase and MDM2 E3-ubiquitin ligase [18, 22]. Therefore, oocyte SIRT1 is considered to be essential for gametogenesis, arguably including oocyte meiotic maturation, for fertilisation and subsequent embryogenesis [17, 23, 24]. Additionally, the study of SIRT1 in oocyte meiosis provides relevant knowledge of the cell cycle of general medical significance.

In this study, our observations point out the involvement of SIRT1 in oocyte meiosis via epigenetic and non-epigenetic factors, based on affected targets. These results are the first to describe the benefits of a specific SIRT1 activator, BML-278, for the chromatin integrity of non-interphase cells through the revealed molecular mechanism. Moreover, our experiments show that pharmacological SIRT1 activation is a possible way to improve the viability of oocytes.

Methods

Animals

All animal procedures were conducted in accordance with Act No. 246/1992 Coll., on the Protection of Animals against Cruelty, under supervision of the Animal Welfare Advisory Committee at the Charles University, Faculty of Medicine in Pilsen, and approved by the Animal Welfare Advisory Committee at the Ministry of Education, Youth and Sports of the Czech Republic.

Six- to eight-week-old ICR female mice were maintained in a facility with a 12 h light: 12 h dark photoperiod, a temperature of 21 ± 1 °C and a relative humidity of 60% and had free access to food and water throughout the period of the study. Females were administered with i.p. 5 IU PMSG, and the experiment was terminated 48 h later for isolation of immature GV (germinal vesicle) oocytes. To obtain *in vivo* mature oocytes, PMSG-treated females were administered with 5 IU hCG, and cumulus-oocyte complexes were flushed from oviducts 16 h later.

Chemicals

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA), if not otherwise stated. BML-278 (Abcam, Cambridge, UK; Cat. No. ab144536), a selective SIRT1 activator (EC_{50} = 1 μmol/L vs. EC_{50} 25 and 50 μmol/L for SIRT2 and SIRT3, respectively), and sirtinol, selective SIRT1 and SIRT2 deacetylase inhibitor (Abcam, ab141263), were used in this study. Moreover, BML-278 activity was compared with resveratrol (Abcam; ab120726), non-selective sirtuin activator, using a fluorometric SIRT1 Activity Assay Kit (Abcam; ab156065), in accordance with manufacturer's instructions.

In vitro maturation

Ovaries were dissected and immature fully grown oocytes at GV stage were isolated and manipulated in M2 medium supplemented with 100 μmol/L isobutylmethylxanthine (IBMX). Fully grown and cumulus cell-free GV oocytes with intact ooplasm were placed into M16 medium containing 100 μmol/L IBMX for 1 h, followed by *in vitro* maturation in IBMX-free M16 for 16 h at 37 °C and 5% CO_2. For the elucidation of the SIRT1 activation effect on the quality of mature oocytes, the culture medium was supplemented with BML-278 to final concentrations of 0.125, 0.25 and 0.5 μmol/L during oocyte meiosis progression. Alternatively, GV oocytes were treated with BML-278 in M16-IBMX for 16 h, and the effect of SIRT1 activation on GV chromatin was studied. In all treatment studies, BML-278 was dissolved in DMSO, and its concentration in M16 did not exceed 0.1% (*v*/*v*), therefore, a vehicle control (VC) consisting of 0.1% DMSO was included. Concurrently, untreated *in vitro*-matured oocytes were incubated with 10 μmol/L Taxol (in 0.1% DMSO, *v*/*v*), an anti-microtubule depolymerising agent, for 45 min at 37 °C. All oocytes were processed for immunocytochemistry as described below.

Fixation and immunocytochemistry

Oocytes at all stages were fixed in two ways: either i) in 4% paraformaldehyde in PBS with 0.1% polyvinyl-alcohol (PVA), 30 min for at room temperature, or alternatively, ii) for H3K9me3, H3K4me2 imaging, in PFA-TX-100 for 15 min, at 37 °C, following permeabilisation in 0.03% Tween 20 in PBS-PVA for 60 s at 37 °C. Subsequently, all oocytes were equally permeabilised in PBS containing 0.04% Triton X-100 and 0.3% Tween 20, for 15 min. Thereafter, oocytes were blocked in 1% BSA in PBS with Tween 20 for 15 min. The 1 h incubation of oocytes with specific antibodies (all diluted 1:200, if not otherwise noted) followed: anti-SIRT1 (Abcam; ab104833; 1:200), anti-SIRT2 (Abcam; ab51023, 1:100), anti-α tubulin (Cell Signaling Technology, Leiden, Netherlands; #2144; 1:200), anti-acetylated α-tubulin (Abcam; ab24610; 1:200), anti-H3K9me2/3 (Abcam; ab184677; 1:200), anti-H3K4me2

(Abcam; ab7766; 1:200), and anti-ubiquitinated (K119) H2A (H2AK119ub; Cell Signaling Technology; D27C4; 1:200). Thereafter, washing and 1 h incubation with the cocktail of anti-mouse-AlexaFluor 488 and anti-rabbit-AlexaFluor 647 (1:200), respectively, were used. Concurrently with washing after the cocktail of secondary antibodies, phalloidin (Thermo Fisher Scientific, Waltham, MA, USA; 1:200) was applied for 15 min for β-actin visualisation. Stained oocytes were mounted onto slides in a Vectashield medium with 4′6′-diamidino-2-phenylindole (DAPI; Vector Laboratories Inc., Burlingame, CA, USA). Images were acquired using spinning disk confocal microscope Olympus IX83 (Olympus, Germany) and VisiView® software (Visitron Systems GmbH, Germany).

TUNEL assay

Fixed oocytes were permeabilised in 0.1% Triton X-100 in PBS containing 0.05% NaN_3 for 40 min. Oocytes were treated with fluorescein-conjugated dUTP and the terminal deoxynucleotidyl transferase enzyme (In Situ Cell Death Detection Kit, Cat. No. 11684795910, Roche, Mannheim, Germany), for 1 h in the dark at 37 °C, in accordance with the assay protocol. Positive control (PC) was prepared using DNase I kit (AMP-D1, Sigma-Aldrich). Finally, oocytes were mounted as mentioned above, and chromatin was visualised. Images were acquired as described above.

Image analysis and colocalisation

Negative controls were performed by omitting specific antibodies and these slides were processed at comparable settings. Immuno- and TUNEL-stained oocytes were subjected to measurement of, integrated density' (expressing signal intensity) of appropriate colour channels using ImageJ software (NIH, Bethesda, CA, USA). Nuclear signal intensities were scaled by signal intensity of corresponding ooplasms. Thereafter, the values of integrated density were related to control oocytes (VC = 1). JACoP (Just Another Co-localisation Plugin) approach for colocalisation of SIRT1 with spindle α-tubulin was used (according to Bolte and Cordelieres [25]). The Costes' randomisation (Costes' rand), modifying Pearson's coefficient R_r according to Costes et al. [26], and Manders' overlap coefficients (R, $M1$ and $M2$) were used for estimation of colocalisation and overlap. Colocalisation analysis was performed on oocyte spindles used as the region of interest (ROI).

Western blotting

Oocytes were collected and lysed in Laemmli buffer containing Triton-X-100 (0.003%, *v*/*v*) and SDS (0.001%, *v*/*v*), enriched with Complete Mini Protease Inhibitor Cocktail (Roche, Switzerland). Samples were boiled and subjected to SDS-PAGE electrophoresis in precast gradient gels and blotted using Trans-Blot TurboTM Transfer System onto a PVDF membrane (Bio-Rad Laboratories, Steenvoorde, France). After blocking in 5% non-fat milk in TBS with 0.5% Tween-20 (TBS-T) overnight at 4 °C, the membrane was incubated with mouse monoclonal anti-SIRT1 (1:1,000). Mouse monoclonal anti-β-actin loading-control antibody (Santa-Cruz Biotechnology, Inc., UK; sc-47778; 1:1,000) was used under the same conditions. Subsequently, the membrane was incubated with horseradish peroxidase (HRP)-conjugated goat anti-mouse or anti-rabbit IgG in TBS-T (Thermo Fisher Scientific; 1:10,000) for 1 h at room temperature. Proteins with adequate molecular weight were detected using the ECL Select Western Blotting Detection Reagent (GE Healthcare Life Sciences, Amersham, UK) and visualised by ChemiDocTM MP System (Bio-Rad Laboratories, Steenvoorde, France).

Statistical analysis

Data from three independent experiments were processed and analyzed. Because of their significant non-normality (Shapiro-Wilk test) the data are represented by medians with appropriate quantiles and a non-parametric method, i.e. Kruskal-Wallis ANOVA, was used for the comparison of the study groups. In case of a significant overall finding, the differences between individual group pairs were assessed by a post hoc test, using multiple comparisons of mean ranks. Because of an asymmetry of the data distribution, logarithmic scale was used in the boxplots. The data were processed with Statistica Cz 12 (StatSoft, USA). The level of statistical significance was set at $\alpha = 0.05$ and two-tailed P values are indicated.

Results

SIRT1 re-localisation during the progress of oocyte meiosis

In this experiment, we immunolabelled SIRT1 and described its subcellular localisation in mouse oocytes matured *in vitro*. For better visualisation of mature oocytes and their meiotic progress, α-tubulin and β-actin were co-immunolabelled. SIRT1 was exclusively located in germinal vesicles (GVs) of immature oocytes, and only a weak signal in ooplasm was obvious. As soon as the meiosis was re-initiated, SIRT1 was dramatically re-localised into the ooplasm of NEBD oocytes. In contrast to the spindles of metaphase I oocytes, where the SIRT1 signal almost disappeared, SIRT1 showed a spindle-like pattern in metaphase II oocytes (Fig. 1a). The binding specificity of anti-SIRT1 antibody (ab104833) against SIRT1 protein (Q923E4, UniProtKB) was verified by primary antibody omitting (Fig. 1b) and confirmed by Western blotting (Fig. 1c). SIRT1 expression was verified

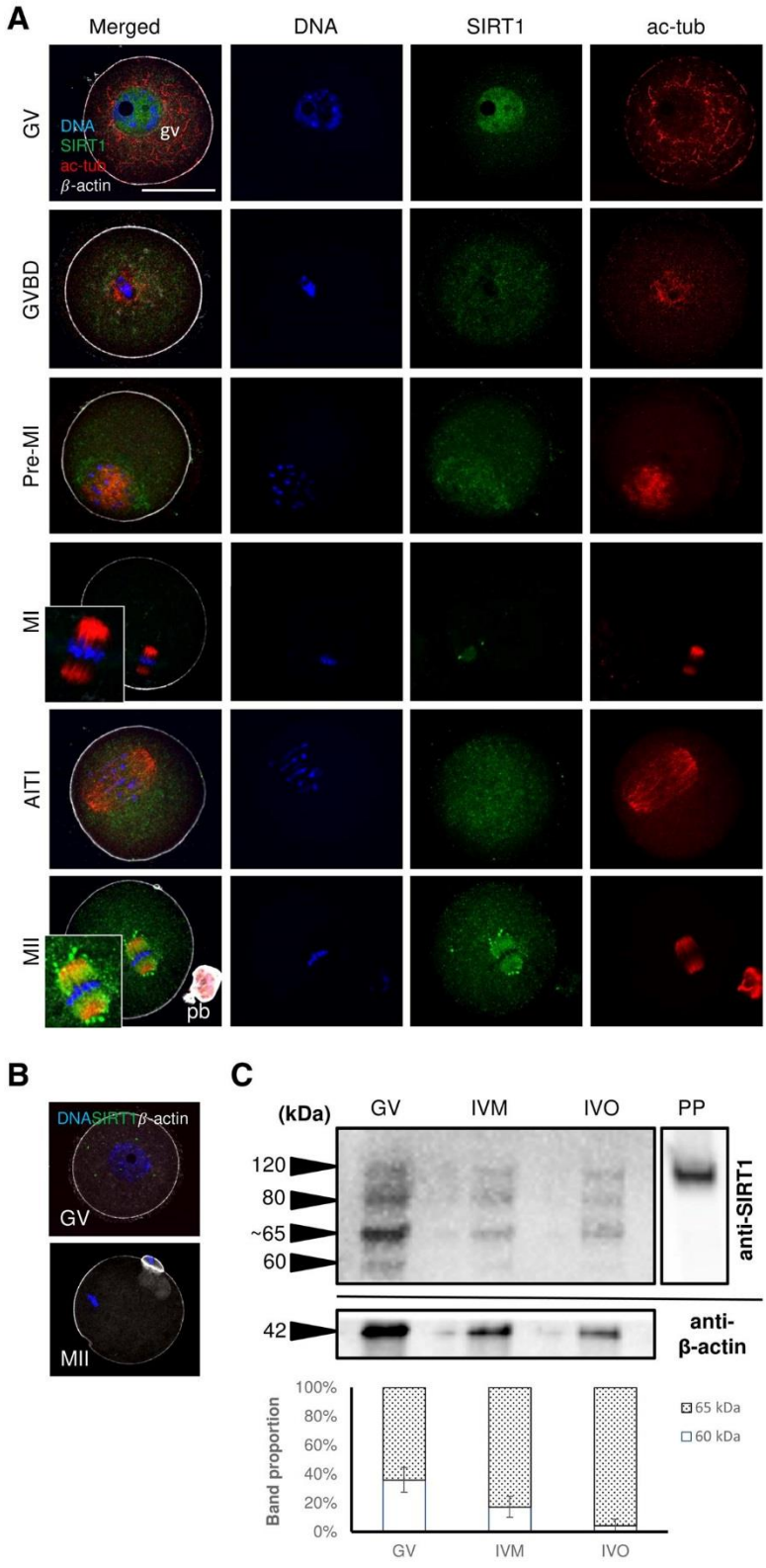


Fig. 1 (See legend on next page.)

(See figure on previous page.)
Fig. 1 SIRT1 subcellular re-localisation during oocyte meiosis. **a** SIRT1 in immature GV oocytes, with visible germinal vesicle (gv), and *in vitro* produced oocytes at different stages, i.e., NEBD (nuclear envelope breakdown), pre-MI (pre-metaphase I), MI, AITI (anaphase-telophase transition) and MII (metaphase II) oocytes with extruded polar body (pb). Scale bar represents 50 μm. **b** Negative control of immunostaining where the primary antibody was omitted. **c** Immunoblotting of SIRT1 (60–120 kDa) and β-actin (42 kDa), in different oocytes (GV, IVM, IVO), including the proportion of 60 and 65 kDa bands (min – max values are indicated). Approximately 200 lysed oocytes were loaded per lane. PP: pure SIRT1 protein. The full-length blot is presented in Additional file 1: Figure S1.3)

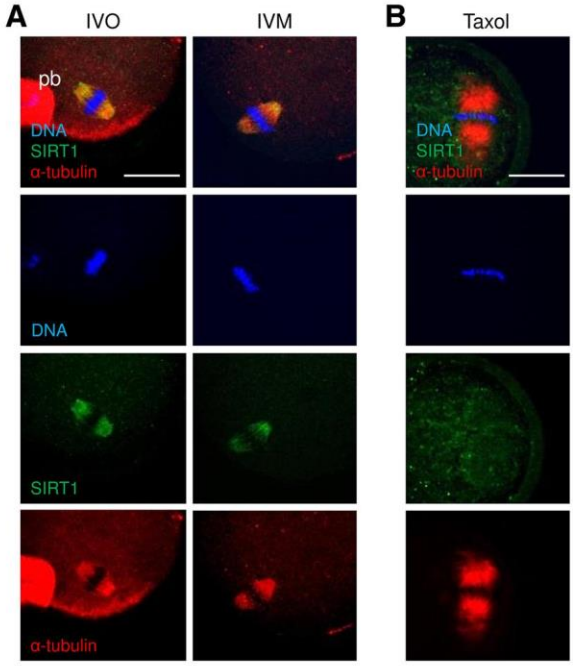
in GV oocytes, and *in vitro* (IVM) and *in vivo* (IVO) matured oocytes. An expected 120-kDa SIRT1 band as well as a ~ 80 kDa one (presumed 75 kDa fragment [27]) were detected, in accordance with the antibody manufacturer and UniProtKB database. Additionally, a 60-kDa and ~ 65 kDa bands were observed, for which a SIRT1 isoform 2 (59.9 kDa) was considered. Interestingly, the 60 kDa bands disappeared in matured oocytes while the 65 kDa bands remained (Fig. 1c). β-actin (42 kDa) was used as an internal standard.

SIRT1 distributes in a spindle-like pattern when the oocyte matured

Based on the SIRT1 subcellular spindle-like pattern observed in the previous experiment, we suggested the association of SIRT1 with cytoskeletal structures in mature oocytes. To support the suggestion of SIRT1-microtubule association, Taxol was used for inhibition of microtubule depolymerisation, followed by co-immunolabelling of both factors. IVO and IVM oocytes subjected to the colocalisation analysis showed a high-level overlap of SIRT1 and α-tubulin on meiotic spindles (Fig. 2a). Moreover, strong SIRT1 association with α-tubulin was detected in IVM oocytes (see Pearson's and Manders' coefficients, used in accordance with previous studies [25, 26]). In contrast to the SIRT1 spindle-like pattern in mature oocytes, Taxol-treated oocytes did not show SIRT1-α-tubulin association, and SIRT1 seemed to be diluted in ooplasm (Fig. 2b). The representative pictures and colocalisation coefficients are summarised in Fig. 2c.

SIRT1 leads to the hypoacetylation of spindle α-tubulin in matured oocytes

Here, the selective SIRT1 activator BML-278 was used. BML-278 was assumed to be highly specific based on the provided manufacture's informations and the biochemical studies known so far [28, 29]. First, we verified the SIRT1 activation capability of BML-278, using a SIRT1 Activity Assay Kit and comparing BML-278 with well-known non-specific SIRT1 activator resveratrol. A comparable activation ability of BML-278 was observed, and there was no significant difference in SIRT1 activity after BML-278 and resveratrol treatments (see Additional file 1: Figure S1.1). Therefore, BML-278 was used for *in vitro* treatment of mature oocytes. The oocyte maturation rate was assessed, oocytes



C

Parameter	IVO	IVM	*P* value
Pearson's coeff.(R_r) by Costes' rand°	0.243±0.030	0.632±0.039	0.000000
Overlap coefficient (r)	0.745±0.009	0.802±0.017	0.000505
Manders' *M1* coeficient	0.439±0.074	0.942±0.020	0.000001
Manders' *M2* coeficient	0.472±0.056	0.835±0.027	0.000003

Fig. 2 Association of SIRT1 with spindle microtubules in matured oocytes. **a** SIRT1 colocalisation with α-tubulin in IVO and IVM matured oocytes. Scale bar represents 25 μm. **b** Co-immunolabelling of SIRT1 and α-tubulin in Taxol-treated IVO oocytes. **c** The results of colocalisation analysis in IVO and IVM oocytes. JACoP (Just Another Co-localisation Plugin) approach for colocalisation of SIRT1 with the spindle α-tubulin was used [25], and sensitivity of Pearson's R_r value to noise and green/red signal intensity variation was eliminated by Costes' randomisation [26] (Costes' rand); the Costes' coefficient modifies Pearson's coefficient estimating automatic threshold, eliminating false-positive colocalisation and signal noise. Manders' overlap coefficient (R) was used for estimation of colocalisation. In addition, Manders' *M1* and *M2* overlap coefficients express the proportion of green (α-tubulin), which is also red (SIRT1), and vice versa, respectively, with respect to spindle localisation as the ROI. N (number of analysed matured oocytes) = 15 per group. *t*-test was used and *P* values are indicated

were subsequently immunolabelled, and the acetylation of spindle α-tubulin in mature metaphase II oocytes was quantified. No effect of BML-278 on the meiosis progress and maturation rate was detected (Additional file 1: Figure S1.2). We observed the decline in acetylated α-tubulin after 0.25 μmol/L BML-278 treatment (Fig. 3b). On the other hand, sirtinol (selective SIRT1 and SIRT2 deacetylase inhibitor) increased signal intensity of acetylated α-tubulin (Fig. 3c). Based on SIRT1 and SIRT2 colocalization (Additional file 1:Figure S1.4), we consider these findings as a result of SIRT1 action. With respect to the SIRT1 pattern in mature and Taxol-treated oocytes mentioned above, we explain it by a temporary limited deacetylating action of SIRT1 on tubulin during oocyte spindle formation, rather than after spindle establishment.

SIRT1-modulated epigenome of mature oocytes

Although SIRT1 is exclusively immunolocalised on the spindles of matured oocytes, the epigenetic SIRT1 action in oocytes is considered in accordance with our own previous findings [18]. Therefore, several post-translational histone modifications, such as positive and negative markers of genome stability, i.e., H3K9me3 [31] and H3K4me2 [32], were analysed as previously described. Moreover, we established H3K9me3 as a double-marker of SIRT1 action: i) direct histone H3 deacetylation, and ii) indirect histone H3 methylation of the same lysine residue [18]. In addition to already well-known histone markers, ubiquitinated (K119) H2A (H2AK119ub) was analysed because a SIRT1 overlap with ubiquitin-associated proteins has been reported [33]. Moreover, there have been contradictory findings of actual association of H2AK119ub with eu- or heterochromatin markers [34, 35], and H2AK119ub significance for mature oocyte quality remain unknown. Finally, the DNA protective effect of SIRT1 activator BML-278 was elucidated with a TUNEL (terminal deoxynucleotidyl transferase dUTP nick) assay.

The fold-change in signal intensity of trimethylation of histone H3 at lysine K9 (H3K9me3) after 0.25 μmol/L BML-278 treatment increased compared to control oocytes (Fig. 4a, d). The pericentric H3K9me3 pattern, described in previous studies [36, 37], was verified using chromosome

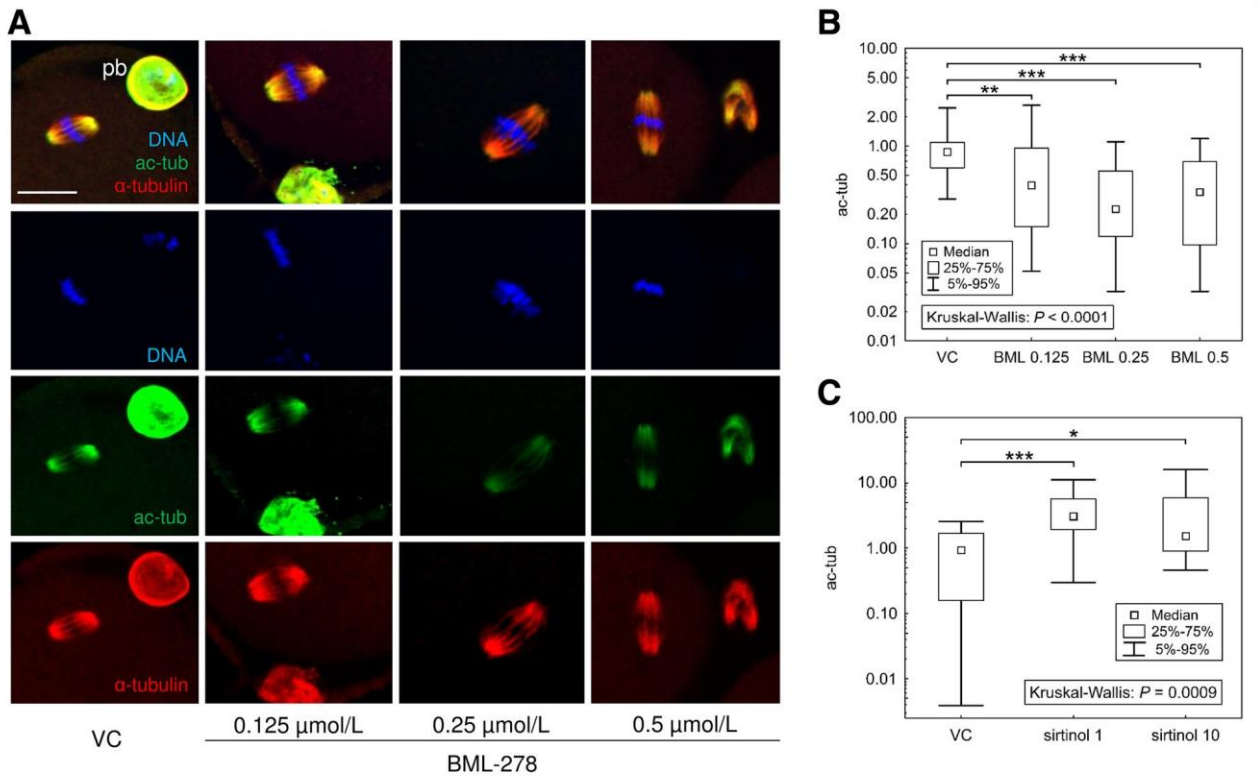


Fig. 3 Deacetylation of spindle α-tubulin in IVM oocytes after SIRT1 activation. **a** Representative images of immunolocalised acetylated α-tubulin (ac-tub) in BML-278-treated oocytes. Scale bar represents 25 μm. **b** Image analysis of ac-tub in IVM oocytes. The quantification of integrated density parameter via ImageJ software was performed. The integrated density of BML-278-treated oocytes is normalised to the mean of the signal in vehicle control oocytes (VC) and expressed as a median ± quantiles from five independent experiments ($N \geq 35$ per group). **c** Quantification of integrated density of oocytes treated with sirtinol (1 and 10 μmol/L), a SIRT1 inhibitor. Differences between individual group pairs were assessed post-hoc using multiple comparisons of mean ranks [30], including a Bonferroni adjustment for multiple testing; *, ** and *** denote significance at $P \leq 0.05$, 0.01 and 0.005, respectively

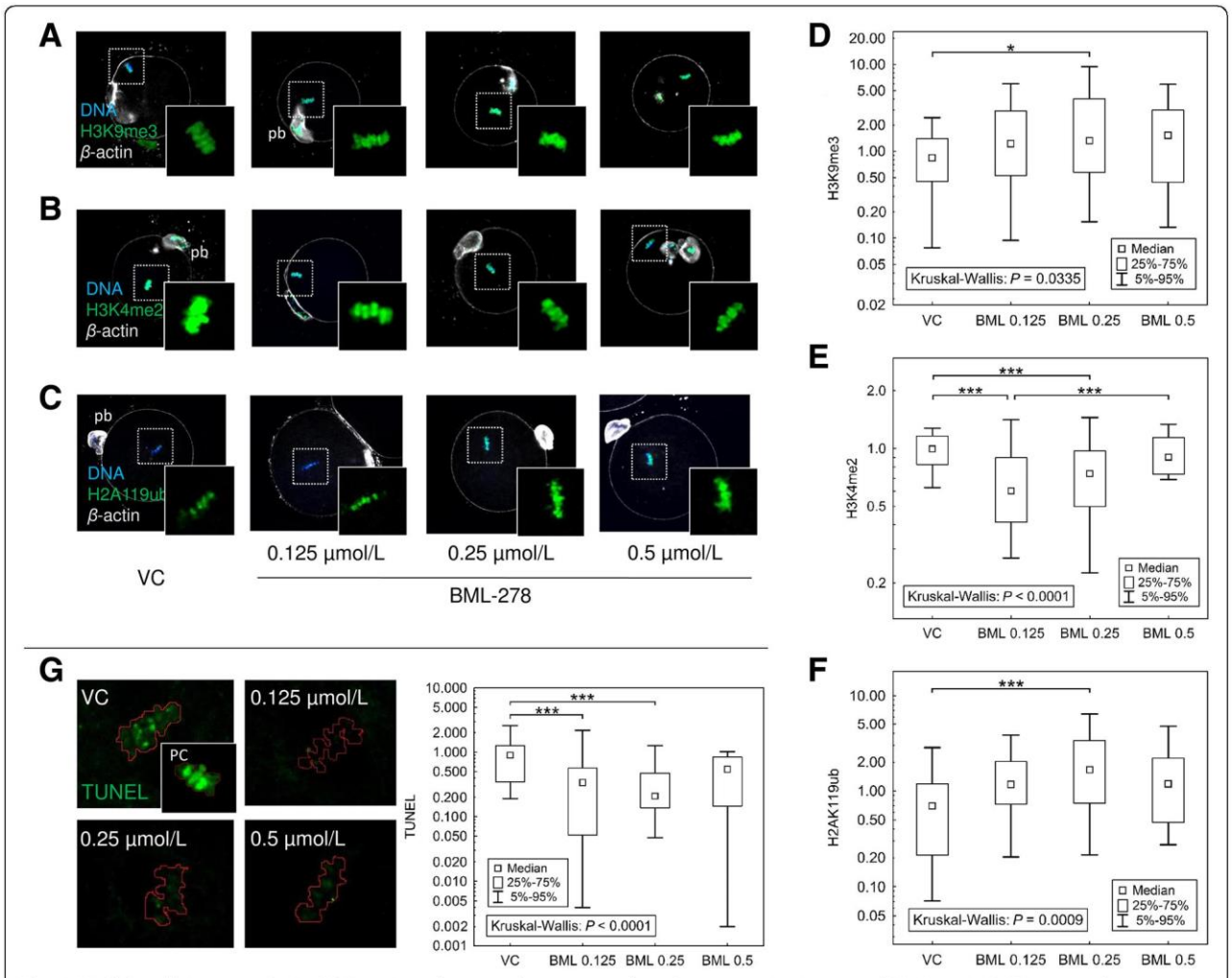


Fig. 4 Modulated histone code in SIRT1-activated matured oocytes. **a**, **b**, **c** Representative images of H3K9me3, H3K4me2 and H2AK119ub, respectively, and the emphasis of metaphase area, used for the quantification of integrated density parameter via ImageJ software. **d**, **e**, **f** Quantification of integrated densities of H3K9me3, H3K4me2 and H2AK119ub, respectively, localised and analysed on metaphase MII chromosomes. Integrated densities are normalised to the mean of vehicle control oocytes (VC) and expressed as a median ± quantiles from five independent experiments ($N \geq 30$ oocytes per group). **g** Effect of SIRT1 activation on DNA damage. TUNEL signal was detected in mature oocytes after BML-278 treatment, and integrated density was quantified ($N \geq 30$ oocytes per group). The region of interest (ROI) for TUNEL signal was established in accordance with DNA staining (red line on representative pictures). TUNEL assay was elucidated using positive control (PC) through DNase I digestion of oocyte chromatin. Differences between individual group pairs were assessed post-hoc using multiple comparisons of mean ranks [30], including a Bonferroni adjustment for multiple testing; * and *** denote significance at $P \leq 0.05$ and 0.005, respectively

spreading and co-staining with centromere-associated Kinesin-13 protein KIF2A (Additional file 1: Figure S1.5). In contrast, signal of dimethylation of H3 on K4 (H3K4me2) was significantly decreased to 0.69 and 0.74 after 0.125 and 0.25 μmol/L BML-278 treatments, respectively (Fig. 4b, e). The increase in H2AK119ub was etected after 0.25 μmol/L BML-278 treatment (2.46 ± 0.33 versus 1.0 ± 0.17; Fig. 4c, f), consistent with the H3K9me3 heterochromatin marker. The DNA protective effect of BML-278-activated SIRT1 was assessed through TUNEL assay. In accordance with H3K9me3 and H3K4me2, we observed decreasing integrated TUNEL density in oocytes matured in the presence of BML-278 (0.125 and 0.25 μmol/L), compared to control oocytes (0.36–0.47 vs. 1.0; Fig. 4g).

Our results showed significant changes in signal intensities of the fluoresceins staining individual histone PTMs. These findings point out the SIRT1-shifted histone code and chromatin quality of matured oocytes after BML-278 treatment. Moreover, the heterochromatin-associated ubiquitination of H2A, rather than as a DNA damage marker is strongly indicated. Although SIRT1 lost the

association with chromatin as soon as NEBD occurred, we rendered SIRT1-driven chromatin quality in matured MII oocytes, and therefore, we assumed that SIRT1 modulates histone code in immature GV oocyte because SIRT1 is exclusively localised in GVs.

SIRT1 drives histone code establishment in immature GV oocytes

In this experiment, GV oocytes were kept under meiosis-suppressing conditions for 16 h and treated with SIRT1 activator BML-278. With respect to the exclusive SIRT1 location in GVs, we assumed histone targets of SIRT1 in GV immature oocytes. To test this suggestion, we used previously introduced histone markers, and the integrated densities of H3K9me3 and H3K4me2 were analysed. We observed an increase in the signal intensity of H3K9me3 after 0.25 μmol/L and 0.5 μmol/L BML-278 (2.91 ± 0.83 and 4.84 ± 1.16, respectively, vs. 1.0 ± 0.15 in control). In contrast, these BML-278 doses had no effect on H3K4me2, however, 0.125 μmol/L BML-278 treatment decreased the signal intensity of H3K4me2 (Fig. 5). Based on the observed effect of BML-278 treatment on signal intensities of both histone PTMs, we can consider SIRT1 epigenetic mode of action in immature GV oocytes and a different, rather non-epigenetic, molecular mechanism in mature oocytes. These findings are in accordance with a previous observation, where SIRT1 was lacking in the perichromatin area immediately after NEBD, and colonised spindles in matured MII oocytes.

Discussion

Sirtuins represent a potent group of proteins relevant to several fields of medical studies, including both veterinary reproduction and human assisted reproduction technologies [23, 38–40]. Indeed, in accordance with previous studies [41–43], we detected SIRT1 in both immature GVs and matured metaphase II (MII) oocytes through different approaches. In addition to SIRT1 detection, the possibility of modulation of SIRT1 activity due to pharmacological treatment offers many medical implications. Therefore, we have chosen BML-278, a SIRT1 specific activator with only minor ability to activate SIRT2 and SIRT3. Based on recent knowledge, BML-278 is the most selective activator for SIRT1 [28, 29]. Moreover, observed phenotypes are considered to be a

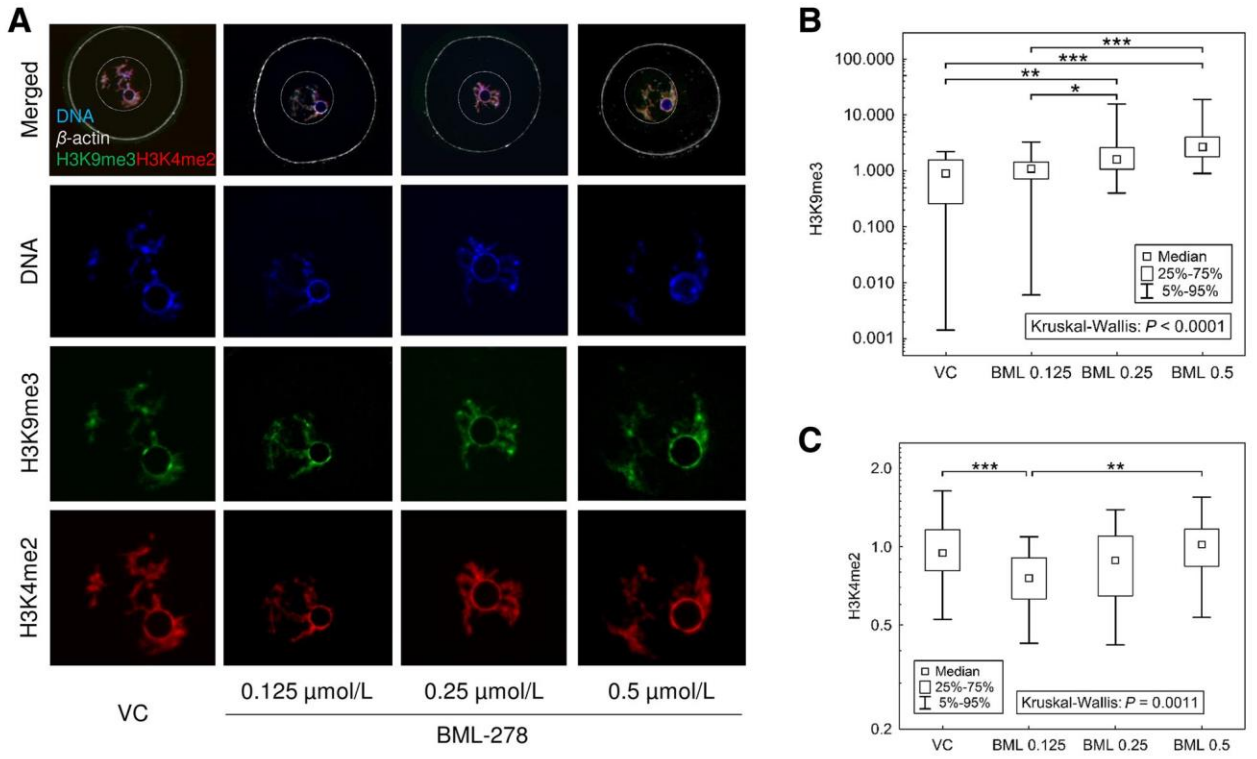


Fig. 5 Modulated histone code in BML-278-treated immature GV oocytes. **a** Representative images of H3K9me3 and H3K4me2 subjected to the quantification of integrated density parameter via ImageJ software. **b**, **c** Quantification of integrated density of H3K9me3 and H3K4me2 localised and analysed in germinal vesicle (GV) of oocytes treated with BML-278 activator, respectively. The integrated density of BML-278-treated oocytes is normalised to the mean of the signal in control oocytes and expressed as a median ± quantiles from five independent experiments ($N \geq 30$ oocytes per group Differences between individual group pairs were assessed post-hoc using multiple comparisons of mean ranks [30], including a Bonferroni adjustment for multiple testing; *, ** and *** denote significance at $P \leq 0.05$, 0.01 and 0.005, respectively

result of SIRT1 action with respect to different subcellular localisation of SIRT1 and SIRT2.

Our observations reveal SIRT1 to be present exclusively in GV of immature oocytes; however, the SIRT1 signal is diluted in the ooplasm immediately after nuclear envelope breakdown (NEBD), and finally forms a spindle-like pattern in matured MII oocytes, comparable with other histone deacetylases in oocytes [44–46] and SIRT1 in human somatic cells [47]. Based on this finding, we have elucidated the deacetylating action of SIRT1 towards spindle tubulin in matured MII oocytes that was proved by sirtinol (a SIRT1 deacetylase inhibitor) treatment. Our study, utilising colocalisation analysis, quantified SIRT1 association with the spindle α-tubulin and revealed the overlap of both factors in *in vivo* and *in vitro* matured oocytes. This observation is in accordance with the previously described involvement of HDAC3 and HDAC8 in deacetylation of spindle tubulin, which, therefore, is responsible for microtubule attachment to the kinetochore and euploidy maintenance in matured oocytes [44, 45]. Even, SIRT2 has been found on the spindle, however, in contrast to our observation, SIRT2 occupies uniquely metaphase I spindle [48]. Surprisingly, the deacetylating action of HDACs on the spindle tubulin is considered a phenomenon essential for metaphase II spindle assembly in oocytes [44, 46], although tubulin acetylation is a marker of stable microtubules [49]. On the other hand, SIRT1 may contribute to microtubule polymerisation via alternative PLK1 regulation [50] through equal localisation on the oocyte spindle.

The SIRT1 spindle pattern observed in mature oocytes seems to be a result of successive SIRT1 re-localisation and short-term spindle occupation. This suggestion is supported by i) almost no observable signal in metaphase I oocytes and subsequently, a gradual spindle-like pattern of SIRT1 during meiosis progression, ii) no association of SIRT1 with overpolymerised α-tubulin in Taxol-treated oocytes, and iii) weaker association of SIRT1 in *in vivo* mature oocytes. Accordingly, *in vivo* mature oocytes represent a physiological control for *in vitro* experiments, and lower colocalisation coefficients underline decreasing SIRT1 requirements on oocyte spindles after metaphase II achievement. Based on the findings in *in vivo* matured oocytes and Taxol-treated oocytes, declining SIRT1-α-tubulin association suggests a preparation of the spindles of mature oocytes for subsequent changes following fertilisation [51]. Moreover, other SIRT1-associated proteins and potent deacetylating targets (e.g., transcriptional factors, core histones) are worth considering [18, 52, 53], and a complex physiological role of the SIRT1 action through these substrates remains to be elucidated.

Because of the well-known SIRT1 targets leading to modulation of the epigenetic code [18, 33], we studied several histone modifications in mature MII oocytes treated after SIRT1 activation. In accordance with the aforementioned postulation of many SIRT1 targets in the oocyte, we revealed the SIRT1-shifted histone code towards chromatin stabilisation and DNA protection, in accord with other sirtuins [54, 55]. We may consider both epigenetic and non-epigenetic substrates in oocyte epigenome modulation, such as lysine K9 in histone H3 deacetylated through SIRT1 and enzymes catalysing methylation of core histones, respectively. Considering histone methylation, increasing activity of SUV39H1 histone methyltransferase is suggested, in accordance with previous observation [22], to result in increased H3K9me3 [56]. In contrast, histone demethylase LSD1/KDM1A demethylating H3K4 is a plausible substrate of SIRT1 in mouse oocytes [21]. Therefore, SIRT1 can achieve significant impact through the regulation of LSD1/KDM1A in gametes as well as early embryos and embryonic stem cells [57, 58].

In addition to H3K9me3 and H3K4me2, we elucidated the ubiquitination of H2A at lysine K119 (H2AK119ub). In our experiment, BML-278 treatment increases H2AK119ub signal although decreasing occurrence is assumed, in accordance with the knowledge of ubiquityl-H2A accompanying DNA damage [35, 59]. However, there is also an evidence of H2AK119ub to be heterochromatin repressive mark [34, 60]. In accordance with these studies, we consider an involvement of SIRT1 in ubiquitin-proteasomal system-modulated chromatin, which is consistent with our earlier findings of MDM2 ubiquitin E3 ubiquitin ligase interaction with SIRT1 [18]. A dual physiological role of H2AK119ub seems to be heterochromatin-marking and DNA protection in mature oocytes, however, a comprehensive study is required for testing of this hypothesis.

The above-mentioned histone PTMs occur in mature oocytes, although SIRT1 subcellular localisation is not associated with condensed chromosomes. These facts lead us to postulate an inheritance of histone modifications acquired earlier than oocytes mature. Therefore, we tested post-translational changes of histone H3 in GV oocytes after SIRT1 activation via BML-278. Indeed, GV histone H3 is modified at lysine K9 in a SIRT1-dependent manner, favouring heterochromatin features for gene silencing, chromatin stability and DNA protection [61, 62]. In addition to H3K9me3, H3K4me2 shows a decrease after 0.125 μmol/L BML-278 treatment of GV oocytes. Hence, we suggest that the SIRT1-modulated histone code, observed in mature oocytes, is attained earlier and inherited from the GV stage. Furthermore, the involvement of LSD1/KDM1A demethylase may be considered in SIRT1-driven modulation of H3K4me2 in GV and MII oocytes [21]. This assumption is supported by the observation of LSD1 spindle-like distribution in somatic cells [63], and we can surmise the SIRT1-LSD1

crosstalk resulting in modulation of H3K4me2 in mature oocytes.

SIRT1 seems to be capable of both epigenetic and non-epigenetic mode of molecular action, in immature GV oocytes and matured MII oocytes, respectively. Accordingly, immature GV oocytes, arrested in the first meiotic arrest, tender more available chromatin for epigenetic modulators in extensive time window, including oocyte growth [64, 65]. On the other hand, mature oocytes arrested at a time-limited stage of metaphase II and containing highly condensed chromatin offer fewer opportunities for epigenetic modifications. The ability to switch the epigenetic and non-epigenetic mode of action during oocyte maturation is proposed and the mechanism of this exchange is a subject of further study.

In accordance with the epigenetic to non-epigenetic switch assumption, anti-SIRT1-immunodetected 60-kDa protein (supposed SIRT1 isoform 2) shows a shift in molecular weight of this protein towards 65-kDa with oocyte maturation. The change indicates an achievement of post-translational modification [66–68] and/or protein-protein interaction [69], in mature MII oocytes. Based on our best knowledge, we presumed a crosstalk of SIRT1 and the ubiquitin-proteasomal system [33, 66]. A description of SIRT1-interacting proteins and clarification of the physiological role of SIRT1 PTMs in oocyte maturation, fertilisation and early embryonic development remains to be elucidated.

Conclusions

Our results show that SIRT1 is predestined for an epigenetic mode of action in immature GV oocytes while SIRT1 distributes in a spindle-like pattern in fully mature oocytes where SIRT1-decreased tubulin acetylation occurs. Our observations suggest a dual spatiotemporal SIRT1 action in oocytes and the capability of being readily switched during the meiosis progress is indicated.

Additional file

Additional file 1: Figure S1.1. The effect of BML-278 on SIRT1 activity. **Figure S1.2.** The results of oocyte maturation after BML-278 treatment. **Figure S1.3.** The full-length blot of SIRT1 and β-actin (loading control). **Figure S1.4.** SIRT2 expression in oocytes and colocalisation with SIRT1. **Figure S1.5.** H3K9me3 and KIF2A co-immunostaining of chromosome spread. (DOCX 2033 kb)

Abbreviations

BML-278: a selective SIRT1 activator; GV: Germinal vesicle; H2AK119ub: Ubiquitinated histone H2A on lysine K119; H3K16ac: Acetylated histone H3 on lysine K16; H3K4me2: Di-methylated histone H3 on lysine K4; H3K9me3: Tri-methylated histone H3 on lysine K9; HDAC: Histone deacetylase; IBMX: 3-Isobutyl-1-methylxanthine; IVM: *In vitro* maturation (of oocytes); IVO: *In vivo* ovulated (oocytes); KIF2A: Kinesin family member 2A, centromere-associated kinesin-like protein ; LSD1/KDM1A: Lysine-specific histone demethylase 1A/lysine (K)-specific demethylase 1A; MDM2: Mouse double minute 2 homolog, E3 ubiquitin-protein ligase ; MII: Metaphase II (oocytes); NEBD: Nuclear envelope breakdown; PLK1: Polo-like kinase 1, serine/threonine-protein kinase 1; SIRT, Sirtuin: Silent mating type information regulator 2 homolog, NAD^+-dependent histone deacetylases; SUV39H1: Suppressor of variegation 3-9 homolog 1, histone-lysine N-methyltransferase; TUNEL: Terminal deoxynucleotidyl transferase (TdT) dUTP nick-end labeling assay

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Authors' contributions

JN, LL, JM, SP, EK and TF performed experimental procedures. MS, PH, HR and TF analysed and interpreted data. JN, PK, KH, TZ, JP and MK designed experiments, interpreted results and prepared the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

Please contact author for data requests.

Ethics approval and consent to participate

All animal procedures were conducted in accordance with Act No 246/1992 Coll., on the Protection of Animals against Cruelty, under supervision of the Animal Welfare Advisory Committee at the Charles University, Faculty of Medicine in Pilsen, and approved by the Animal Welfare Advisory Committee at the Ministry of Education, Youth and Sports of the Czech Republic (approval ID MSMT-11925/2016–3).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Increased Expression of Maturation Promoting Factor Components Speeds Up Meiosis in Oocytes from Aged Females

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Abstract: The rate of chromosome segregation errors that emerge during meiosis I in the mammalian female germ line are known to increase with maternal age; however, little is known about the underlying molecular mechanism. The objective of this study was to analyze meiotic progression of mouse oocytes in relation to maternal age. Using the mouse as a model system, we analyzed the timing of nuclear envelope breakdown and the morphology of the nuclear lamina of oocytes obtained from young (2 months old) and aged females (12 months old). Oocytes obtained from older females display a significantly faster progression through meiosis I compared to the ones obtained from younger females. Furthermore, in oocytes from aged females, lamin A/C structures exhibit rapid phosphorylation and dissociation. Additionally, we also found an increased abundance of MPF components and increased translation of factors controlling translational activity in the oocytes of aged females. In conclusion, the elevated MPF activity observed in aged female oocytes affects precocious meiotic processes that can multifactorially contribute to chromosomal errors in meiosis I.

Keywords: aging; oocyte; MPF; meiosis; translation; lamin A/C

1. Introduction

The development of female germ cells (oocytes) is essential for sexual reproduction. Oocytes, arrested in meiotic prophase, undergo a major growth phase during their development in ovarian follicles. During this phase, they actively transcribe their genome; however, most derived mRNAs are stored in ribonucleoprotein particles to be used much later during the final stages of meiosis and early embryonic development. A unique property of the oocyte is that the final stages of meiosis (after prophase I) occur in the absence of de novo transcription. Consequently, regulation of mRNA stability and translation serves as the main driving forces behind oogenesis and early embryogenesis [1].

Mammalian oocytes undergo two successive cell divisions without an intermediate replicative phase. This brief period is called "meiotic maturation" and is crucial for the formation of an egg capable of being fertilized and for the generation of viable and euploid offspring. At the onset of meiosis I, the nuclear lamina is phosphorylated (namely lamin A/C; LMN A/C) and disassembled, leading to nuclear envelope break down (NEBD), chromosome condensation, and progressive reorganization of microtubules into a bipolar spindle [2]. At the end of meiosis I, the first asymmetric division occurs.

Human and mouse oocytes are vulnerable to aging as the incidence of chromosome segregation errors (aneuploidy) reaches high levels in females/mothers of advanced age [3–5]. For example, in 20-year-old women, aneuploidy occurs in ~2% of matured oocytes; however, after 35 years of age aneuploidy increases to 35% [6,7]. Similarly, oocytes from aged mice display a significant increase in the incidence of aneuploidy. In three-month-old mice, aneuploidy occurs in 5% of cases; however, by 12 months of age this figure increases to 30–50% [4,8,9]. The majority of chromosome segregation errors are known to arise during the first meiotic cytokinesis [6,10]; however, the reasons why female meiosis shows this peculiar vulnerability to aging remains unclear.

In this study, we present evidence for the aberrant timing of meiosis I in the oocytes derived from female mice of advanced age. Such age-associated abnormalities present as aberrations in nuclear envelope morphology as well as the precocious timing of NEBD and the formation of kinetochore-microtubule (K-MT) attachments, resulting in accelerated first polar body extrusion (PBE). Furthermore, we reveal that it is the overexpression of metaphase promoting factor (MPF) components associated with impaired translational machinery that leads to this phenotype.

2. Results

2.1. Meiosis I Is Accelerated in Oocytes from Females of Advanced Age

It is well known that increased maternal age negatively affects oocyte quality [3,5]. We isolated oocytes from antral follicles and obtained an average of 22 fully grown GV oocytes per young mouse (YF; 2 months old) compared to 3 oocytes per aged female (AF; 12 months old). Following removal of IBMX from the culture medium, to restart meiosis I, 98.75% of selected oocytes from young females and 98.53% oocytes from aged females resumed meiosis (NEBD; Student's t-test $p = 0.9985$). Of the cells that resumed meiosis, 84% of the young oocytes extruded polar body and reached MII in the 12 h period compared to 94% of AF oocytes (Student's t-test $p = 0.010809$). Measurement of oocyte diameter did not show any differences between age groups (71.73 $\pm$ 1.5 and 72.31 $\pm$ 1.6 μm, respectively, Student's t-test $p = 0.99743$). To analyze the effect of maternal age on the progress of meiosis I, we compared the maturation of mouse oocytes from young females (YF; 2 months old) and aged females (AF; 12 months old). Time-lapse microscopy revealed that the oocytes from AF progress through meiosis I significantly 30 min faster than oocytes from YF ($p < 0.05$; Figure 1a,b). The oocytes in the AF group initiate nuclear envelope breakdown (NEBD) earlier (Figure 1a) and consequently polar body extrusion (PBE) also occurs earlier than in the YF group ($p < 0.05$; Figure 1b); manifest as a shortening of time between NEBD and PBE (Figure 1b). Next, we scored the attachment of individual cold-stable microtubules (MT) with end-on kinetochores in both age groups. We found that, during metaphase I, 6 h after releasing oocytes from prophase I (6 h post-IBMX-wash), the AF group had a higher number of stably attached kinetochores (95.5%) than the YF group (75.8%, $p < 0.01$) (Figure 1c,d). The larger number of stably end-on attached kinetochores in the AF group demonstrates that the progression through meiosis I was accelerated in the oocytes from the AF group.

2.2. Dissociation of Nuclear Envelope Is Accelerated in the Oocytes from Aged Females

The abundant components of the nuclear envelope are lamin A and C (LMN A/C) [11]. Phosphorylation of these lamins at Serine-22 (Ser22) triggers the disassembly of the nuclear lamina, which is a prerequisite for nuclear envelope breakdown [12]. Therefore, we analyzed the phosphorylation of LMN A/C (Ser22) as a marker of meiotic progression. Oocytes from both age

groups were analyzed by Western blotting at various time points relative to their initial isolation (i.e., after 0, 1, 3, 6, and 12 h). We found that the AF group had a significantly increased level of phosphorylated LMN A/C 1 h post-IBMX-wash ($p < 0.05$; Figure 2a,b). On the contrary, the YF group only had an abundant level of phosphorylated LMN A/C 3 h post-IBMX-wash (Figure 2a,b). Despite the observed different timing of LMN A/C phosphorylation between these two groups, the total/eventual level of LMN A/C remained constant (Figure 2a,c).

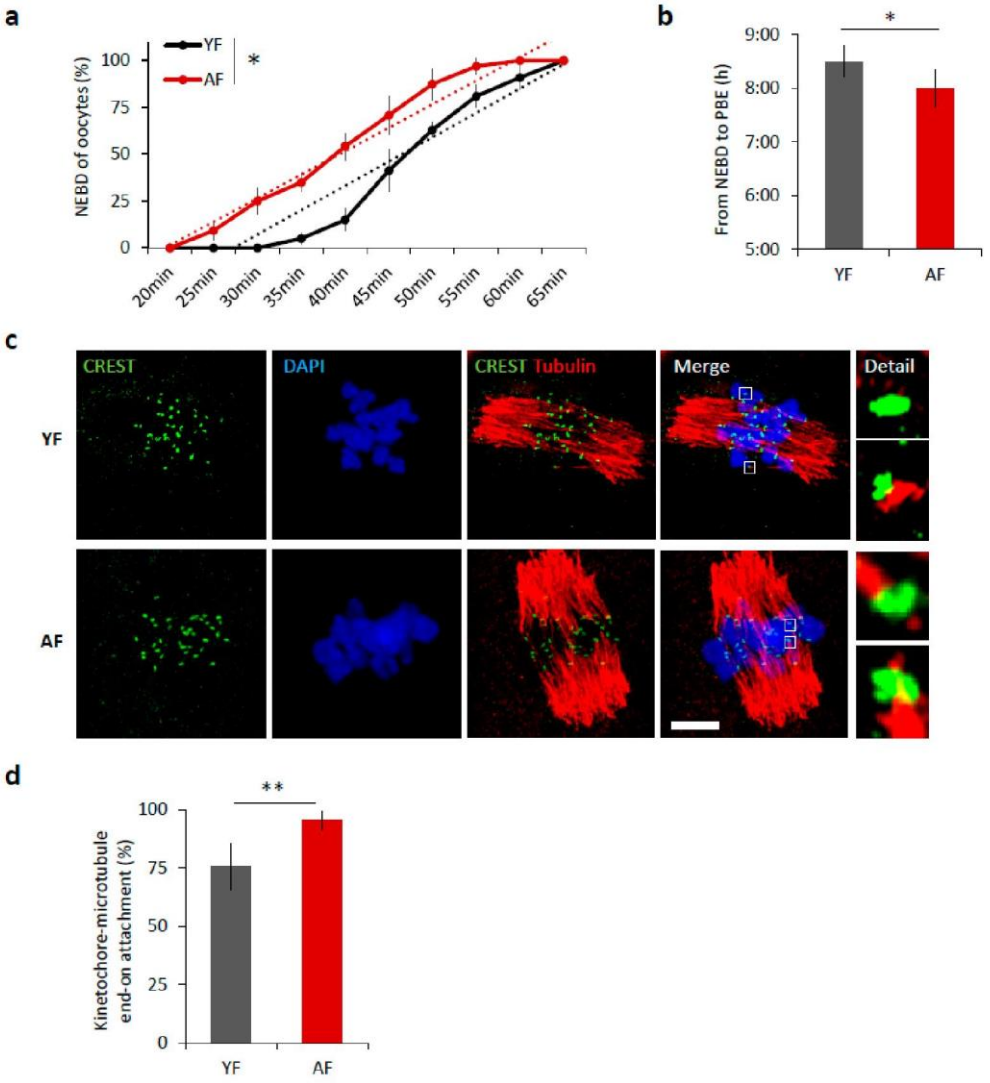

Figure 1. Meiosis I is accelerated in oocytes from females of advanced age. (**a**) Timing of nuclear envelope breakdown (NEBD) of oocytes isolated from young females (YF, $n = 80$; black line) and aged females (AF, $n = 68$; red line). Trend line is depicted by dot line. Data represent mean ± SD, $n = 6$, * $p < 0.05$, Student's *t*-test. (**b**) Time from NEBD to polar body extrusion (PBE) in oocytes from YF ($n = 80$; t = 8:30 h) and AF ($n = 68$; t = 8 h). Data represent mean ± SD and data are from at least three experiments of biologically different samples. * $p < 0.05$, Student's *t*-test. (**c**) Representative Z-projections from the assessment of cold stable attachments of kinetochore (KT) to microtubule (MT) imaged by confocal microscopy using CREST (green) and Tubulin (red) antibodies. Representative images from three experiments of biologically different samples are presented (scale bar, 10 μm). (**d**) The percentage of cold stable end-on MT to KT attachments in each age group averaged over multiple cells ($n \geq 15$) 6 h post-IBMX-wash. Kinetochore-MT end-on attachments were quantified. The morphology of kinetochores analyzed is specified in detail. Data represent mean ± SD. ** $p < 0.01$, Student's *t*-test.

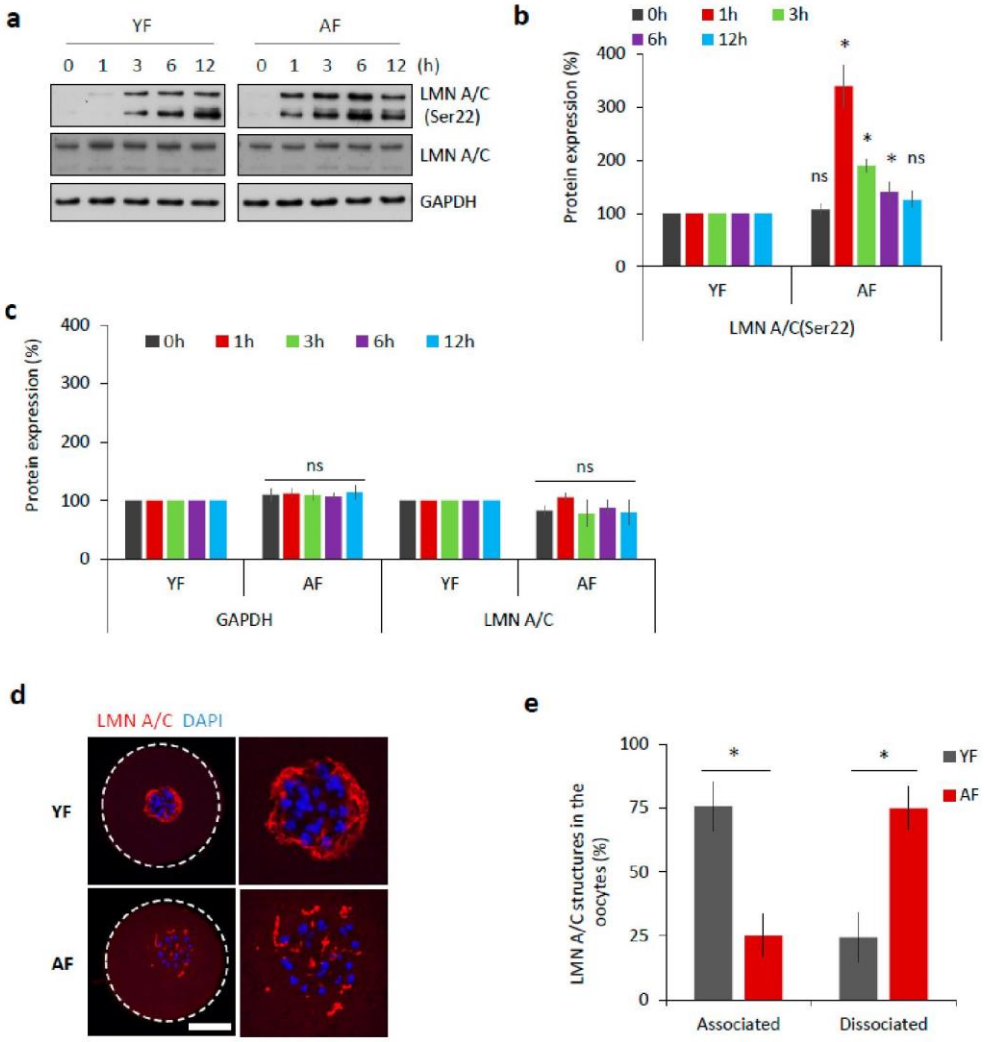

Figure 2. Dissociation of nuclear lamina is accelerated in the oocytes from the AF group. (**a**) Western blot analysis of phosphorylation status of LMN A/C (Ser22) at different time points during meiotic progression (0 h, GV; 1 h, post-NEBD; 3 h, post-NEBD; 6 h, post-NEBD/metaphase I; 12 h, post/NEBD/metaphase II). Antibodies against LMN A/C and GAPDH were used as loading controls. Representative images are from three experiments of biologically different samples. (**b**) Quantification of LMN A/C phosphorylation (Ser22) at different time points during meiotic maturation. Data are from three experiments of biologically different samples. Values obtained for the YF group were set as 100%. AF values from each antibody was compared between groups and same oocyte stage. Data represent mean ± SD. * $p < 0.05$, bars with ns are non-significant, Student's *t*-test. (**c**) Quantification of GAPDH and LMN A/C protein expression at different time points during meiotic maturation. Data are from three experiments of biologically different samples. Values obtained for the YF group were set as 100%. AF values from each antibody was compared between groups and same oocyte stage. Data represent mean ± SD. * $p < 0.05$, bars with ns are non-significant, Student's *t*-test. (**d**) Representative images of LMN A/C structures 3 h post-IBMX-wash (post-NEBD, scale bar 20 μm). The cortex of the oocyte indicated by the white dashed line. See Figure S1 for the LMN A/C localization and phosphorylation during oocyte meiotic progression and Figure S2 for electron microscopy images of the nuclear lamina. (**e**) Quantification of LMN A/C structures in the oocytes from different age groups post-NEBD ($n \geq 33$ and three independent biological replicates). Data represent mean ± SD. * $p < 0.05$, Student's *t*-test.

It has been previously documented [13,14] that nuclear lamina structures can be still present at least a few hours after NEBD in mouse oocytes. By immunocytochemistry (ICC), we visualized LMN A/C structures during oocyte meiotic maturation (Figure S1a,b). Using specific antibodies,

both total LMN A/C as well as phosphorylated LMN A/C (Ser22) were detected within the disrupted nuclear lamina structures in NEBD stage oocytes, 3 h post-IBMX-wash (Figure S1a,b). The observed lamina structures surrounded the chromosomal area, where the new spindle was due to be assembled (Figure S1c), but disappeared as meiosis progressed (Figure S1). When we compared 3 h post-IBMX-wash oocytes from both age groups, we found that the dissociation of the described LMN A/C structures was completed significantly faster in the AF group, at a time-point at which they still persisted in the YF oocytes ($p < 0.05$; Figure 2d,e).

Additionally, we imaged GV stage oocytes (oocytes with intact nucleus designated as germinal vesicle, GV) from both age groups by transmission electron microscopy and we were able to distinguish visible differences in the structure of the nuclear envelope in both groups. The nuclear membrane of AF oocytes presented an unique characteristic series of invaginations and decreased compactness (Figure S2a,b). The distinct morphology of the nuclear envelope in the AF oocyte group resulted in a significant increase in the circumference of the nuclear envelope ($p < 0.01$; Figure S2b). Moreover, the observed ultrastructural morphology of the nuclear lamina in AF oocytes resembled that reported in the nuclear phenotypes of other aged cells [15,16].

To conclude, in addition to the above-mentioned precocious timing in meiosis, observed in AF oocytes, we also observed a comparatively earlier phosphorylation of LMN A/C that was associated with a faster disassembly of nuclear lamina, thus affecting the timing of nuclear membrane breakdown, when compared to oocytes from the YF group.

2.3. CDK1 Activity Is Responsible for NEBD in Mouse Oocytes

NEBD is reported to be driven by CDK1 (MPF) activity via phosphorylation of lamin proteins and subsequent lamina disassembly at the onset of meiotic resumption or mitosis [17,18]. To test whether LMN A/C were phosphorylated in a CDK1-dependent manner, we treated mouse oocytes with 20 μM Roscovitine (Rosco), a potent inhibitor of CDK1 activity, for 2 h after NEBD. We found significantly decreased levels of LMN A/C (Ser22) phosphorylation in oocytes treated with Rosco ($p < 0.05$; Figure 3a,b) versus controls, a result that is consistent with findings of [17].

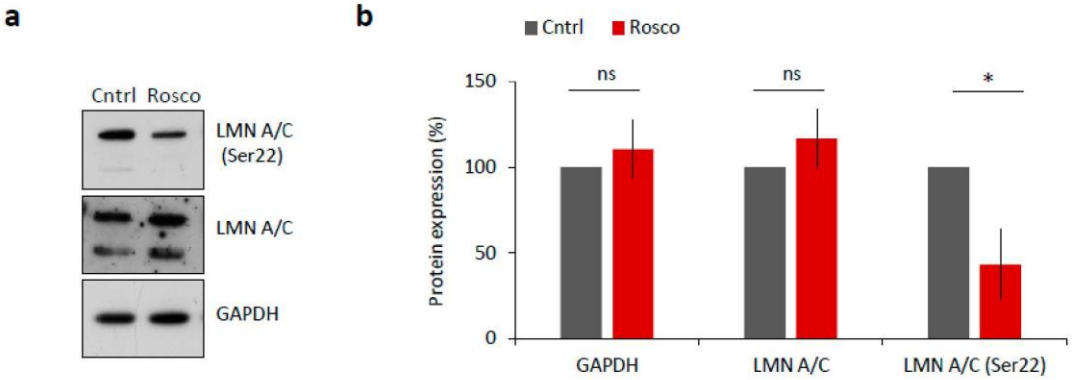

Figure 3. CDK1 is responsible for LMN A/C phosphorylation in mouse oocyte. (**a**) Western blot analysis of oocyte samples treated for 2 h after NEBD with 20 μM CDK1 inhibitor, Roscovitine (Rosco). Phosphorylation status of LMN A/C (Ser22) was detected using a specific antibody. Antibodies against LMN A/C and GAPDH were used as a loading control. (**b**) Quantification of total and phosphorylated LMN A/C after Roscovitine (Rosco) treatment. Protein levels were normalized in a way that non-treated controls are 100%. Data was derived from three experiments containing biologically different samples. Columns represent mean, ± SD; ns non-significant; * $p < 0.05$, Student's *t*-test.

Thus, our data confirm the functional involvement of the activated MPF in nuclear lamina disassembly through regulation of LMN A/C phosphorylation status.

2.4. CDK1 Activity Is Increased in Mouse Oocytes from Aged Females

We next examined, whether the expression of MPF components, that directly affect meiotic progression [19], differs between the YF and AF groups of mouse oocytes. Firstly, we isolated total RNA from transcriptionally silent GV staged oocytes from each group and performed quantitative RT-PCR mRNA expression analysis of the MPF component genes *Cdk1* and the B-type *Cyclins*. We found significantly increased levels of both *Cdk1* and *Cyclin B* transcripts in the oocytes from the AF group (Figure 4a) that were not reflected in the total RNA content (Figure S3a) nor in the expression level of *Gapdh* mRNA (Figure 4a). Next we analyzed the expression of MPF components at the protein level via Western blotting, and again we discovered a significant increase in the expression levels of CCNB and CDK1 proteins, specifically in the AF group of oocytes (Figure 4b,c). In addition to the use of a pan-CDK1 antibody, we also probed the oocyte samples with an antibody that specifically recognized phosphorylated (Thr161) CDK1, the enzymatically active form of the protein that is required for a functional MPF activity [19–22]. Again, we found increased phosphorylation status of CDK1 in the AF group versus the YF group of oocytes (Figure 4d,e). Consistently, an analysis of MPF activity, using a standard kinase assay, also revealed a significant increase in CDK1 activity at 1 h in the AF oocytes compared with the YF oocytes, at the time of NEBD (Figure 4d,e).

Taking these results together, we conclude that the activation of the MPF is significantly accelerated in the oocytes from aged females.

2.5. Translation of Positive Regulators of Translation Is Increased in the Oocytes from Aged Females

De novo transcription in fully grown oocytes ceased, so we wondered whether the elevated levels of CCNB and CDK1 proteins in the oocytes from aged females were only due to higher transcript abundance or could also be related to higher translational activity. To experimentally address this question, we compared the incorporation of ^{35}S-Methionine into nascently translated proteins in both the YF and AF groups of oocytes during maturation (Figure S3b), but we found no significant difference in the levels of global translation (Figure S3b,c). However, we also derived an RNA-seq dataset of mRNA polyribosomal occupancy that allowed us to detect and identify actively translated mRNAs in the two studied age groups of oocytes. Whilst the polysome occupancy was unchanged for mRNAs encoding GAPDH, CDK1, and CCNB1, we did intriguingly identify *Ccnb2*-derived transcripts enriched over 11-fold in polysomal fractions from AF compared to YF oocytes (Figure 5a). A further GO-term (gene ontology) enrichment analysis of polysome-bound mRNAs indicated a significant enrichment of mRNA coding for protein factors belonging to GO functional categories associated with translation initiation and regulation, specifically in the AF oocyte group (p-value = $4.76-6.34 \times 10^{-6}$ for 38 individual mRNAs) (Figure 5b). Generally, this enrichment was higher and the respective categories contained more mRNAs in the AF group over the YF oocytes. We therefore systematically examined the polysome-bound mRNAs whose products are involved in the regulation of translation and revealed increased levels of mRNA coding for positive translation regulators, namely, eukaryotic translation initiation factors (eIF2D, eIF3E, eIF4B, eIF4E3, and eIF4G1), polyadenylation factors (PABPN1L and PABPN1), elongation factor (eEF2), and the number of ribosomal proteins (60S-RPL6, RPL10, RPL10A, RPL17, RPL19, RPL23A, RPL24, RPL37, RPL38; 40S-RPS6, RPS8, RPS9, RPS13, RPS16, and RPS25) in the AF group of oocytes (Figure 5c). Contrarily, we detected a decreased level of mRNA-polysome association for the elongation factor kinase (eEF2K), which is known to act as a suppressor of translational elongation (Figure 5c).

Overall, these results suggest that the translation of individual MPF components and of specific translational factors is elevated in AF oocytes, which is likely to result in changes in the physiology of oocytes from aged female mice.

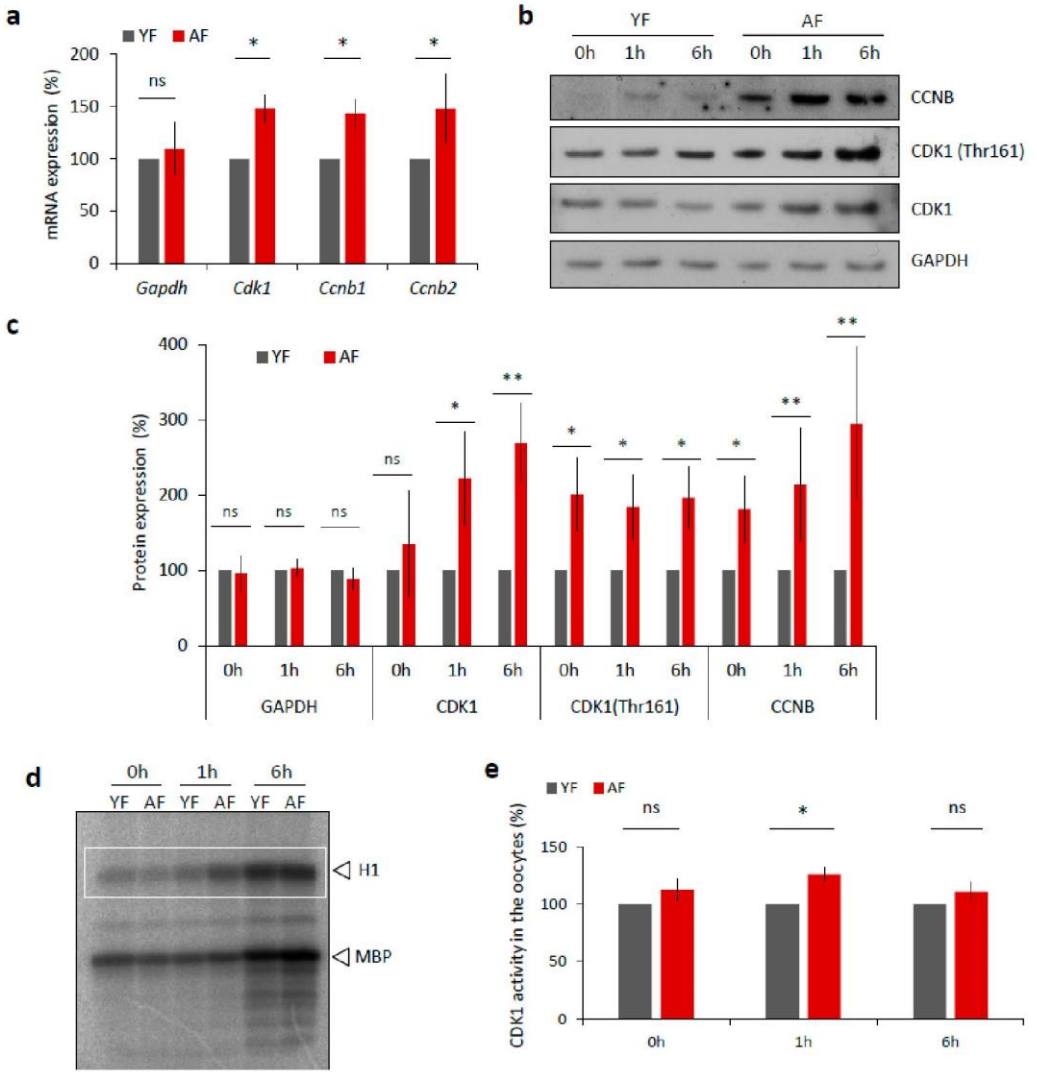

Figure 4. Expression of MPF components and its activity is increased in the oocytes from aged females. (**a**) RT-PCR quantification of mRNA coding for CDK1 and B-type cyclins, as well as loading control *Gapdh* in the GV oocytes (0 h) from different age groups. For quantification of total RNA content in oocytes from YF and AF groups see Figure S3a. Values obtained for the YF group were set as 100%. Data was derived from at least four experiments of biologically different samples. Columns represent mean; error bars ± SD; ns non-significant; * $p < 0.05$, Student's *t*-test. (**b**) Western blot analysis of CDK1, CDK1 (Thr161) and CCNB during oocyte maturation (0 h, 1 h and 6 h) in the both age groups. See Figure S3b,c for the assessment of global translation in oocytes from YF and AF groups. (**c**) Quantification of MPF components, CDK1, its phosphorylation (Thr161), CCNB and GAPDH as a loading control. Values obtained for the YF group were set as 100%. From at least three experiments of biologically different samples. Columns represent mean ± SD; * $p < 0.05$; ** $p < 0.01$; bars with ns are non-significant; Student's *t*-test. (**d**) Representative image of analysis of CDK1 activity (H1) in the oocytes after isolation (0 h), NEBD (1 h) and at metaphase I (6 h). Kinase assay was done with oocytes of both female age groups. CDK1 activity was measured towards histone H1 as external substrate, marked by white rectangle. (**e**) Quantification of CDK1 (H1 substrate) activity during oocyte maturation from YF and AF groups. Measurements originated from four experiments of biologically different samples. Values obtained for the YF group were set as 100%. Columns represent mean; error bars ± SD; ns non-significant; * $p < 0.05$; Student's *t*-test.

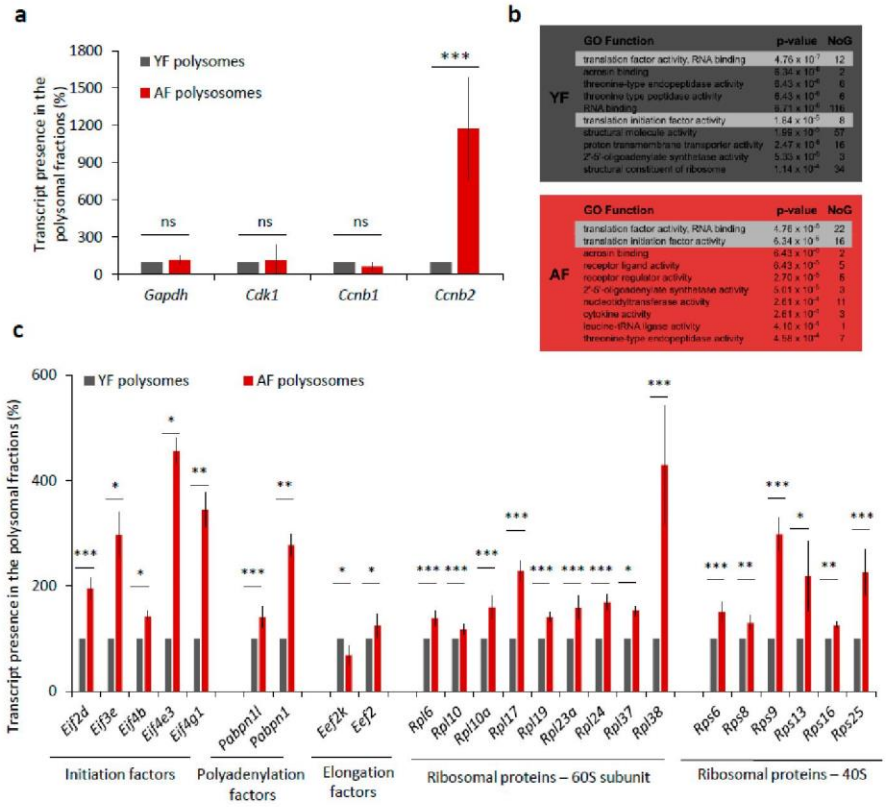

Figure 5. Translation of a number of translational factor components is increased in the oocytes from aged females. (**a**) mRNA abundance of MPF components, *Cdk1* and *Cyclins B* as well as *Gapdh* used as the control in the polyribosomal fractions. Percentage of reads from RNA-Seq. See Figure S3a for quantification of total RNA in oocytes from YF and AF groups. Three independent experimental datasets from biologically different samples. Values obtained for the YF group were set as 100%. Data columns represent mean; error bars, ± SEM; ns non-significant; *** $p < 0.001$; Student's *t*-test. (**b**) Top 10 most enriched GO Function categories in polysome-bound mRNAs in YF (grey) and AF (red) oocytes determined by Gorilla. NoG (Number of Genes) denotes the number of genes in enriched categories. Highlighted lines represent translational functional categories. (**c**) Translational regulation of mRNA coding for different translational factors from polysomal fractions of YF and AF oocytes. Values obtained for the YF group were set as 100%. Data represent mean ± SEM; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; Student's *t*-test.

2.6. Elevated CDK1 Activity Is Responsible for Faster Meiosis I in Mouse Oocytes

It is known that the expression of CCNB is the limiting factor for the activation of MPF in oocytes prior to resumption of meiosis I [23,24]. It has been reported [25] that the slow increase in CDK1 activity during meiosis I acts as an intrinsic timing mechanism that ensures the appropriate stabilization of kinetochore attachments and thus guards the oocyte against chromosomal segregation errors. We examined whether the overexpression of CCNB affects the timing of meiotic progression. We overexpressed CCNB by microinjecting a cRNA encoding GFP-fused to CCNB into YF oocytes at the GV (0 h) stage (Figure S4). We also microinjected other GV oocytes with *Gfp* cRNA as a negative control. We found that, when experimenting with oocytes derived from the YF cohort, there was a significant increase ($p < 0.05$) in the levels of phosphorylated LMN A/C (Ser22) and phosphorylated CDK1 (Thr161) when microinjected with *Ccnb:gfp* cRNA, as measured 3 h post-IBMX-wash compared to the control group (Figure 6a,b). Live cell imaging of meiotic progression/maturation of such oocytes revealed that the increased expression of CCNB was also able to significantly accelerate overall meiotic progression, as evaluated by the timing of the NEBD and PBE ($p < 0.05$; Figure 6c,d). Specifically, oocytes injected with *Ccnb:gfp* extruded the polar body significantly earlier compared to the control

group (Figure 6c). Thus, they mimicked the phenotype observed in the AF oocytes (Figure 1). Moreover, we also visualized cold-stable kinetochore-microtubule end-on attachments [25,26] and found that the oocytes overexpressing CCNB had a significantly higher rate of the kinetochore-microtubule end-on attachment than the controls at the 6 h post-IBMX-wash ($p < 0.05$; Figure 6e,f). These results suggest that artificially increasing MPF activity leads to a notably more rapid progression through meiosis I as exemplified by the production of stable kinetochore-microtubule attachments.

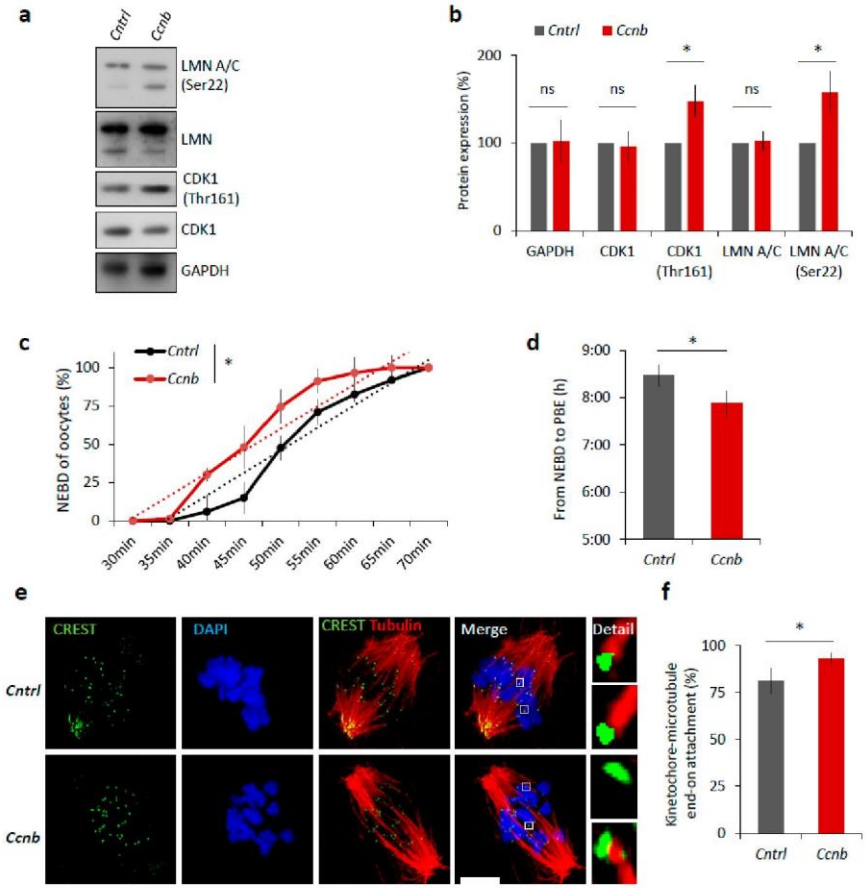

Figure 6. Elevated MPF activity is responsible for meiosis I acceleration in oocytes. (**a**) Western blot analysis of LMN A/C (Ser22), LMN A/C, CDK1 (Thr161), CDK1, and GAPDH in the post-NEBD oocytes (3 h) injected with *Ccnb* RNA or control *Gfp* RNA (*Cntrl*). See Figure S4 for the over-expression of CCNB in oocytes. Representative images from at least three experiments of biologically different samples. (**b**) Quantification of protein expression, LMN A/C, and CDK1 phosphorylation. From three experiments of biologically different samples. Values obtained for the YF group were set as 100%. Data represent mean ± SD; ns non-significant; * $p < 0.05$; Student's *t*-test. (**c**) Timing of NEBD of YF oocytes microinjected with *Gfp* (n = 46; black line) and *Ccnb* cRNA (n = 51; red line). From three experimental data sets of biologically different samples. Trend line is depicted by dot lines. Data represent mean ± SD. * $p < 0.05$, Student's *t*-test. (**d**) Time from NEBD to PBE in oocytes from YF injected with RNA coding for *Gfp* (n = 46; t = 8:25 h) and *Ccnb* (n = 51; t = 7:52 h). Data represent mean ± SD and data are from at least three experiments of biologically different samples. Student's *t*-test. (**e**) Representative Z-projections from the assessment of cold stable attachments of kinetochores (KT, CREST, green) to microtubule (MT, tubulin, red) of oocytes microinjected with control *Gfp* and *Ccnb* cRNA. Representative images are from three experiments of biologically different samples (scale bar, 10 μm). (**f**) The percentage of cold stable end-on MT to KT attachments in each group averaged over multiple cells ($n \geq 28$) 6 h post-IBMX-wash. Kinetochore-MT end-on attachments were quantified. The morphology of kinetochores analyzed is specified in detail. Data represent mean ± SD. * $p < 0.05$, Student's *t*-test.

Taken together, these experiments show we were able to mimic the faster meiotic progression observed in the AF oocytes, that itself stems from higher MPF activity.

3. Discussion

Here we have addressed the question of how meiosis I differs in oocytes from females of advanced reproductive age versus those originating from young females. The precise timing of the cell cycle is a prerequisite for the appropriate propagation of genomes. It is well accepted that, in human and mouse oocytes, the incidence of genomic instability and aneuploidy increases with maternal age [5,7,27–30]. Age-associated increase in aneuploidy [27–30] has been attributed, at least in part, to a faster progression through the first meiotic division in oocytes from aged females, which would affect the time available for proper chromosome congression prior to chromosome segregation [27]. The mouse is a useful model in which to study the effect of age on egg quality, including the molecular basis for observed age-associated increase in aneuploidy.

We have found that oocytes from aged females resume meiosis and progress through meiosis I faster than the oocytes from young females. Our finding is consistent with findings of others [5,27,31–33] but opposes other findings reporting a lack of timing in oocytes from aged females [34,35]. In addition, we found that oocytes from aged females are significantly more meiotically competent than from young females, a further consistent observation [36]. The underlying reasons for the reported discrepancies related to the length of meiosis I in aged oocytes are not clear; however, they may have their origin in the methodologies employed to select meiotically competent GV oocytes and/or further differences in oocyte manipulation (e.g., the removal of cumulus cells and microinjection). Relating to our own data, we conclude that an increase in MPF activity during meiosis I temporary regulates acceleration of NEBD, the attachment of chromosomes, and cytokinesis events in aged oocytes. Moreover, our results are in agreement with previous findings [25] in which it is reported that premature increases of CDK1 kinase activity, induced by cyclin-B microinjection, are responsible for the precocious formation of stable kinetochore-microtubule attachments and lagging chromosomes during anaphase I, a condition that leads to aneuploidy. Thus, the increased presence of MPF components, as observed in our AF oocyte cohort, could clearly act in a similar manner to result in chromosome segregation errors during meiosis I.

Surprisingly, we have also found that transcripts coding for MPF components are significantly overexpressed in the oocytes from aged females. However, it is important to recognize that the abundance of mRNA only represents one part of the regulation of gene expression and that selective translational regulation of transcripts can play a pivotal role. It has been previously reported that CCNB2 has a functional role during the prophase/metaphase transition of mouse oocyte maturation [37], and these data support our findings showing that the increased translational rate of CCNB2 transcripts in the oocytes from aged females might be associated with faster meiotic progression. Consistent with this prevailing view, we have also demonstrated elevated phosphorylation levels of CDK1 (Thr161), that in turn contributes to its MPF activity [38], in AF oocytes. Notwithstanding this observation, the upregulation of Cyclin B clearly plays a positive role in reinforcing CDK1/MPF activity and thus driving meiotic cycle progression. As such, our study correlates the resumption of meiosis with the synthesis of the regulatory subunit of MPF, namely cyclin B1/2, as supported by the fact that the level of MPF activity is known to depend on the amount of cyclin B present [22,39,40]. Thus, the fact that the MPF components in aged GV oocytes are apparently more expressed (but not necessarily fully active) and that they are then rapidly activated during their maturation (in AF versus YF oocytes) contributes to the observed acceleration of the AF oocytes meiotic progression.

We show that both cyclins B are expressed and differentially occupy polyribosomes in the AF group. In addition to the increased expression of MPF components, which leads to accelerated meiotic progression, we also demonstrate that the key components of the translational machinery are more translated (associated with polysome fractions), which in turn is likely to positively affect general translation in AF oocytes. Indeed, our findings are in good agreement with published

data describing increased numbers of ribosomes in oocytes from older females [41]. However, in connection to increased number of ribosomes, we have not observed increased rates of global translation. Nonetheless, our results suggest that there is increased translation of specific mRNAs related to specific translational machinery activity in AF oocytes, which may contribute to amplifying the roles of specific regulators [13,42]; mechanisms that could target specific mRNAs for translation and consequently affect meiotic progression rates. In addition to the increased number of ribosomes and translational regulators in oocytes from females of advanced age, it has also been reported [43,44] that the number of mitochondria is also increased in the oocytes and embryos derived from aged mouse and human subjects.

We have also shown that the GV oocytes from female mice of advanced age have aberrantly formed nuclear envelopes, which strongly resemble the morphology of those in aged somatic cells [15,45]. In association with described precocious meiotic progression and increased MPF activity, we have shown that the nuclear lamina is also precociously dispersed in aged oocytes. We have previously reported that LMN A/C structures surround oocyte chromosomes post-NEBD, resembling an organelle-exclusion "spindle envelope" that acts as a diffusion barrier structure [46–48]. Such spindle envelopes are thought to confine spindle assembly and their mechanical disruption is reported to compromise precise and appropriate chromosome segregation in mitosis [47]. It is therefore possible that the lack of such a functioning spindle envelope in AF-derived oocytes contributes to increased aneuploidy rates observed.

Taken together, our results can provide at least a partial explanation for the commonly recognized multifactorial phenomenon of age-related increase in oocyte aneuploidy on a molecular level. In addition, our study significantly contributes to the overall knowledge base concerning the molecular physiology of aged cells, including but not restricted to oocytes, and provides a solid foundation for further work related to the observed translational discrepancies between young and aged oocytes identified herein, and their functional interplay with meiotic progression/maturation.

4. Material and Methods

4.1. Oocyte Cultivation, Treatment, and Microinjection

GV oocytes were obtained from CD1 mice 46 h after injection by 5 IU pregnant mare serum gonadotropin (PMSG, HOR 272, ProSpec, Rehovot, Israel). Oocytes were obtained from females in two distinct age categories: young females (YF) group (2 months old) and aged females (AF) group (12 months old). Oocytes were isolated in germinal vesicle stage (GV; 0 h) in transfer medium [49] supplemented with 100 μM 3-isobutyl-1-methylxanthine (IBMX, I5879, Sigma-Aldrich, Darmstadt, Germany) to prevent NEBD. Selected fully grown GV oocytes were denuded by pipetting and cultured in M16 medium (M7292, Sigma-Aldrich, Darmstadt, Germany) without IBMX at 37 °C, 5% CO_2. Post-IBMX-wash (PIW) oocytes undergo nuclear envelope breakdown (NEBD) within 1 h; they reach metaphase I in 6 h and metaphase II in 12 h. For oocytes treatment, 20 μM roscovitin (186692-46-6, Cayman Chemical, Ann Arbor, MI, USA) was added 1 h PIW. GV oocytes were microinjected in the presence of the IBMX on inverted microscope Leica DMI 6000B (Leica Microsystems, Wetzlar, Germany) using TransferMan NK2 (Eppendorf, Hamburg, Germany) and FemtoJet (Eppendorf). Oocytes were injected with 20 ng/μL of in vitro transcribed (mMessage, Ambion, Thermo Fisher Scientific, Waltham, MA, USA) RNAs from plasmids (GFP, [50]; CCNB1, Dr. Martin Anger, Laboratory of Cell Division Control, IAPG, CAS) diluted in RNAse free water. Approximately 5 pL of RNA solution were injected into one oocyte. Microinjected oocytes were used for time-lapse microscopy, cold tubulin stability testing, and immunoblotting. All animal work was conducted according to Act No 246/1992 for the protection of animals against cruelty; from 25.09.2014 number CZ02389, issued by Ministry of agriculture.

4.2. Time-Lapse Microscopy

Oocytes were scanned with an inverted wide field microscope, Leica DMI 6000B (Leica Microsystems, Wetzlar, Germany), equipped with a chamber system (Pecon, Erbach, Germany), a Tempcontroller 2000-2 (Pecon), and a CO_2 controller (Pecon). Cover-glass-based 4-well chambers (94.6190.402, Sarstedt, Nümbrecht, Germany) were used for live oocytes imaging. Oocytes were put into a 10 µL drop of M16 medium without IBMX and covered by approximately 1 mL of mineral oil (M8410, Sigma-Aldrich). The chamber was pre-tempered to 37 °C and 5% CO_2. Images were captured every 5 min. Timing of the NEBD and polar body extrusion (PBE) were evaluated through time lapse movies.

4.3. Immunoblotting

Oocytes were washed in phosphate buffer saline (PBS, Sigma-Aldrich) with polyvinyl alcohol (PVA, Sigma-Aldrich) and frozen to −80 °C. An exact number of oocytes (15–30) were lysed in 10 µL of 1× Reducing SDS Loading Buffer (lithium dodecyl sulfate sample buffer NP 0007 and reduction buffer NP 0004, Thermo Fisher Scientific, Waltham, MA, USA) and heated at 100 °C for 5 min. Proteins were separated by gradient precast 4–12% SDS–PAGE gel (NP 0323, Thermo Fisher Scientific) and transferred to Immobilon P membrane (IPVD 00010, Millipore, Merck group, Darmstadt, Germany) using a semidry blotting system (Biometra GmbH, Analytik Jena, Jena, Germany) for 25 min at 5 mA cm^{-2}. Membranes were blocked by 5% skimmed milk dissolved in 0.05% Tween-Tris buffer saline (TTBS), pH 7.4 for 1 h. After a brief washing in TTBS, membranes were incubated at 4 °C overnight with the primary antibodies diluted in 1% milk/TTBS (see Table S1). Secondary antibody Peroxidase Anti-Rabbit Donkey (711-035-152) or Peroxidase Anti-Mouse Donkey (715-035-151, Jackson ImmunoResearch, West Grove, PA, USA) was diluted 1:7500 in 1% milk/TTBS. The membranes were incubated in the secondary antibodies for 1 h at room temperature. Immunodetected proteins were visualized on films using ECL (Amersham, GE Healthcare Life Sciences, Barcelona, Spain). Films were scanned using a GS-800 calibrated densitometer (Bio-Rad Laboratories, CA, USA) and quantified using ImageJ (http://rsbweb.nih.gov/ij/).

4.4. Measurement of Overall Protein Synthesis

To measure the overall protein synthesis, 50 µCi of ^{35}S-methionine (Hartmann Analytics, Braunschweig, Germany) was added to methionine-free culture medium. Ten oocytes per sample were labeled for 2 h, then lysed in SDS-buffer, and loaded to SDS-polyacrylamide gel electrophoresis and transferred to an Immobilon P membrane using the semidry blotting system for 25 min at 5 mA cm^{-2} (the same materials as in Immunoblotting). The labeled proteins were visualized by autoradiography on FujiFilm (incubated at least 14 days in −80 °C), scanned using BAS-2500 Photo Scanner (FujiFilm Life Science, Tokyo, Japan) and quantified by ImageJ. GAPDH antibody was used as a loading control.

4.5. Immunocytochemistry and Cold-Stable MT Assay

After cultivation, oocytes were fixed for 15 min in 4% paraformaldehyde (PFA, Alfa Aesar, Thermo Fisher Scientific, Waltham, MA, USA) in PBS/PVA. Oocytes were permeabilized in 0.1% Triton (X-100, Sigma-Aldrich) in PBS/PVA for 10 min, washed in PBS/PVA, and incubated overnight at 4 °C with primary antibodies (see Table S1). After washing in PBS/PVA, detection of the primary antibodies was performed by cultivation of the oocytes with relevant Highly Cross-Adsorbed Secondary Antibodies, Alexa Fluor 488, 594 or 647 conjugates (Thermo Fisher Scientific) diluted 1:250 for 1 h at room temperature. Oocytes were then washed two times for 15 min in PBS/PVA and mounted using a Vectashield Mounting Medium with DAPI (H-1200, Vector Laboratories, Burlingame, CA, USA). For the cold-stable MT assay, oocytes were matured for 6 h post-IBMX-wash and then were incubated for 15 min in 4 °C, fixed in 4% PFA/PVA, and stained for tubulin and CREST according to the immunocytochemistry protocol. Confocal images were collected as Z stacks at 0.3 µm intervals

to visualize the entire meiotic spindle region. Samples were visualized using a Leica SP5 inverted confocal microscope (Leica Microsystems, Wetzlar, Germany). To classify kinetochore attachment status, images were scored around the same Z plane using the merged two-color confocal stack of CREST and MT images. Images were assembled in software LAS X (Leica Microsystems).

4.6. Transmission Electron Microscopy

Mouse oocytes in GV were washed three times in PBS/PVA and one time in 0.1 M Sorenson's phosphate buffer (pH = 7.2) with PVA. Oocytes were fixed in 2.5% glutaraldehyde (G5882, Sigma-Aldrich) in 0.1 M Sorenson's phosphate buffer for 1 h at room temperature. Fixed oocytes were transported to the Electron Microscopy facility at the Microscopy Centre of the Institute of Molecular Genetics, CAS. Fixative was removed and oocytes were centrifuged (5000× g/5 min) in 1% low-temperature melting agarose. Oocytes were embedded to Epon blocks and sliced by UltraCut6 (Leica Microsystems) to ultra-thin sections. Oocyte sections were imaged at 80 kV using FEI Morgagni 268 Transmission Electron Microscope with Olympus Megaview III Digital Camera EM (FEI Company, Hillsboro, OR, USA).

4.7. RNA Isolation and RT-PCR

RNA was extracted using a RNeasy Plus Micro kit (74034, Qiagen, Hilden, Germany) and genomic DNA was depleted using gDNA Eliminator columns. The quality and quantity of the isolated RNA was analyzed using the Bioanalyzer 2100 (Agilent, Santa Clara, CA, USA) system employing the RNA 6000 Pico kit (5067-1513, Agilent). RT-PCR was then carried out using the Rotor-Gene 3000 (Biocompare, South San Francisco, CA, USA) and the OneStep RT-PCR Kit (210210, Qiagen) and SybrGreen I (S7563, Thermo Fisher Scientific) according to manufacturers' provided protocols. Gene/transcript specific RT-PCR primers were designed with an annealing temperature of 58 °C (see Table S2). The reaction condition for reverse transcription was as follows: 50 °C/30 min, then initial activation at 95 °C/15 min, followed by 40 PCR amplification cycles (95 °C/15 s, 58 °C/20 s, 72 °C/30 s) and 72 °C/10 min. Quantification analyses were performed using a dynamic amplification efficiency determination for each amplification run as provided in the comparative quantification function with the Rotor-Gene RG-3000 software. The exact amplification efficiencies were assessed in each tube, and a mathematic model was applied for the derived calculation of the relative gene expression in the control (YF).

4.8. Polysome Fractionation and RNA Extraction

Prior to oocyte collection, 100 µg/mL of cycloheximide (CHX, 01810, Sigma-Aldrich) was added for 10 min. Next, 200 oocytes (per sample) were washed three times in PBS/PVA supplemented with CHX and frozen at –80 °C in low-binding tube (Eppendorf). To disrupt the zona pellucida and lysate the oocytes, 250 µL of zirconia-silica beads (11079110z, BioSpec, Bartlesville, OK, USA) were added to the tube with frozen oocytes together with 350 µL of lysis buffer (10 mM Hepes, pH 7.5; 62.5 mM KCl; 5 mM $MgCl_2$; 2 mM DTT; 1% TritonX-100; 100 µg/mL of CHX supplemented with Complete-EDTA-free Protease Inhibitor (05 056 489 001 3, Roche Diagnostics GmbH, Mannheim, Germany) and Ribolock 20 U/mL (EO0381, Thermo Fisher Scientific)). Oocytes were disrupted in the mixer mill apparatus MM301 (shake frequency 30, total time 45 s, Retsch, Haan, Germany). Lysates were clarified by centrifugation at 8000× g for 5 min at 4 °C). Supernatants were loaded onto 10–50% linear sucrose gradients containing 10 mM Hepes, pH 7.5; 100 mM KCl; 5 mM $MgCl_2$; 2 mM DTT; 100 µg/mL CHX; Complete EDTA-free (1 tablet/100 mL); and 5 U/mL Ribolock. Centrifugation was carried out using Optima L-90 ultracentrifuge (Beckman Coulter, Brea, CA, USA) at 35,000× g for 65 min at 4 °C. Polysome profiles were recorded using ISCO UA-5 UV absorbance reader. We monitored the overall quality of the polysome fractionation experiment by an inclusion of a parallel HEK293 cells sample. Ten equal fractions were recovered from each sample and subjected to RNA isolation by Trizol reagent (Sigma-Aldrich). Each fraction was then tested by qPCR with 18S and 28S rRNA-specific primers in

LightCycler480 (Roche) to reconstruct a distribution of non-polysomal and polysomal RNA complexes in each oocyte—specific profile.

4.9. Library Preparation, RNA Sequencing and Data Analysis

Fractions corresponding to polysomal and non-polysomal part, respectively, were pulled together. These sub-samples were concentrated to 16 µL of Clean & Concentrator-5 (R1014, Zymo Research, Irvine, CA, USA) and ribosomal RNA was removed from them by Ribozero-Gold (MRZG12324, Illumina, San Diego, CA, USA). Afterwards, RNA was turned into cDNA and amplified by using the REPLI-g WTA Single Cell Kit (150063, Qiagen). Finally, cDNA was tagmented and libraries were prepared using the Nextera DNA Library Prep Kit (FC-121-1030, Illumina). Sequencing was performed in Centro Nacional de Analisys Genomico facility (CNAG, Barcelona, Spain). Samples were sequenced by HiSeq 2500 (Illumina) as 150 bp paired-end. Reads were trimmed using Trim Galore! v0.4.1 and mapped to the mouse GRCm38 genome assembly using Hisat2 v2.0.5. Gene expression was quantified as fragments per kilobase per million (FPKM) values in Seqmonk v1.40.0. Functional annotation was performed using GOrilla [51,52] with ranked lists of genes detected in polysomal fractions (FPKM > 0.1).

4.10. Kinase Assay

Kinase activities of CDK1 (H1) and MAPK (MBP) were determined in a single assay via their capacity to phosphorylate external substrates histone H1 and myelin basic protein (MBP), respectively [53]. Fifteen oocytes per sample were collected and lysed in 5 µL of lysis buffer (10 µg/mL leupeptin, 10 µg/mL aprotinin, 10 mM p-nitrophenyl phosphate, 20 mM β-glycerophosphate, 0.1 mM Na_3VO_4, 5 mM EGTA, 1 mM benzamidine, 1 mM AEBSF) by three cycles of freezing/thawing. Next, 5 µL of double kinase buffer (60 µg/mL leupeptin, 60 µg/mL aprotinin, 24 mM p-nitrophenyl phosphate, 90 mM β-glycerophosphate, 4.6 mM Na_3VO_4, 24 mM EGTA, 2 mM benzamidine, 2 mM AEBSF, 24 mM $MgCl_2$, 0.2 mM EDTA, 4 mM NaF, 1.6 mM DTT, 0.2% (*w*/*v*) polyvinyl alcohol, 40 mM MOPS pH 7.2, 2.2 µM protein kinase inhibitor (P0300, Sigma-Aldrich), 1 mg/mL MBP (M1891, Sigma-Aldrich), 0.5 mg/mL histone H1 (10223549001, Roche), 0.6 mM ATP, 1 mCi/mL [γ-^{32}P] ATP (Hartmann Analytic, Braunschweig, Germany) was incubated with the lysed sample for 30 min at 30 °C. The reaction was terminated by addition of 12.5 µL of double-strength concentrated reducing sample buffer [54]. The phosphorylated substrates were resolved on 15% SDS-PAGE gel, the gel was stained with Coomassie Brilliant Blue R250 (27816, Sigma-Aldrich), dried and exposed to an intensifying screen in the exposure cassette for 20 h. Phosphorylated substrates were visualized using a FujiFilm BAS-2500 Photo Scanner and the kinase activity was quantified using Aida Image Analyzer software (Elysia Raytest, Angleur, Belgium).

4.11. Statistical Analysis

Mean and standard deviation (±SD) values were calculated using MS Excel. Statistical significance of the differences between the groups was tested using Student's *t*-test (PrismaGraph5) and $p < 0.05$ was considered as statistically significant (marked by asterisks: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

Supplementary Materials: Supplementary materials can be found at http://www.mdpi.com/1422-0067/19/9/2841/s1. Figure S1. Localization of LMN A/C during oocyte maturation. (**a**) Representative confocal images from immunocytochemistry (ICC) showed localization of LMN A/C (red) and phosphorylated LMN A/C Ser22 (green) during oocyte maturation (GV 0 h; NEBD 3 h; MI 6 h, MII 12 h). Cortex of oocytes is depicted by white dashed line. DNA, blue and scale bar, 10 µm. (**b**) Co-localization of LMN A/C (Ser22) (green) and the spindle (tubulin, red). DNA, blue and scale bar 10 µm. (**c**) Localization of LMN A/C (red) during oocyte meiotic maturation. DNA, blue and scale bar 10 µm. Arrowhead marks polar body. Figure S2. Transmission electron microscopy of oocyte nuclei from females of different age. (**a**) Representative images of the nucleus from YF and AF oocytes. The images in the right panels show nuclear membrane highlighted with red line. Scale bar 10 µm. (**b**) Measurement of nuclear membrane circumference of oocytes from the YF and the AF group. From two experiments of biologically different samples ($n \geq 8$). Data represent mean ± SD. ** $p < 0.01$, Student's *t*-test. (**c**) Detail of nuclear lamina from AF and YF oocytes. Representative images are from two experiments from biologically different samples (bar, 1 µm).

The images in the right panels show nuclear membrane highlighted with red line. Figure S3. Total RNA amount and global translational activity is not different between YF and AF groups. (**a**) Quantification of total RNA by Agilent 2100 Bioanalyzer in the oocytes from different age groups. From 10 experiments of biologically different samples. Data represent mean ± SD. ns, non-significant, Student's *t*-test. (**b**) ^{35}S-Methionine incorporation during meiotic progression of oocytes from YF and AF groups. Representative images are from three experiments of biologically different samples. (**c**) Quantification of ^{35}S-Methionine incorporation in the oocytes from different groups. From three experiments of biologically different samples. Values obtained for the YF group were set as 100%. Data represent mean ± SD, *ns*, non-significant, Student's *t*-test. Figure S4. Induced expression of the CCNB in the oocytes. Oocytes injected with control *Gfp* (*Cntrl*) and *Ccnb* RNA. See Figure 6a for the effect of the overexpression. WB analysis of samples using CCNB antibody. Arrowhead depicts endogenous CCNB and arrow GFP tagged CCNB protein. GAPDH was used as a loading control. From three experiments of biologically different samples. Table S1. Primary antibodies used for WB and ICC in the study. Table S2. Primers used in the study for RT-PCR.

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Exposure to alternative bisphenols BPS and BPF through breast milk: Noxious heritage effect during nursing associated with idiopathic infertility

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ABSTRACT

There is increasing evidence that bisphenols BPS and BPF, which are analogues of BPA, have deleterious effects on reproduction even at extremely low doses. Indirect exposure *via* the maternal route (*i.e.* across the placenta and/or by breastfeeding) is underestimated, although it can be assumed to be a cause of idiopathic female infertility. Therefore, we hypothesised the deleterious effects of exposure to BPA analogues during breastfeeding on the ovarian and oocyte quality of offspring. A 15-day exposure period of pups was designed, whilst nursing dams ($N \geq 6$ per experimental group) were treated *via* drinking water with a low (0.2 ng/g body weight/day) or moderate (20 ng/g body weight/day) dose of bisphenol, mimicking real exposure in humans. Thereafter, female pups were bred to 60 days and oocytes were collected. Immature oocytes were used in the *in-vitro* maturation assay; alternatively, *in-vivo*-matured oocytes were isolated and used for parthenogenetic activation. Both *in-vitro*- and *in-vivo*-matured oocytes were subjected to immunostaining of spindle microtubules (α-tubulin) and demethylation of histone H3 on the lysine K27 (H3K27me2) residue. Although very low doses of both BPS and BPF did not affect the quality of ovarian histology, spindle formation and epigenetic signs were affected. Notably, *in-vitro*-matured oocytes were significantly sensitive to both doses of BPS and BPF. Although no significant differences in spindle-chromatin quality were identified in ovulated and *in-vivo*-matured oocytes, developmental competence was significantly damaged. Taken together, our mouse model provides evidence that bisphenol analogues represent a risk to human reproduction, possibly leading to idiopathic infertility in women.

1. Introduction

Human infertility has become a serious medical issue in developed countries, due to increased maternal age (Bukovsky, 2017), lifestyle practices (Leisegang and Dutta, 2020), and/or environmental threats (Di Renzo et al., 2015). However, many infertility cases have been classified as 'idiopathic', when the primary cause is unknown (Monteiro et al., 2020; Punab et al., 2016). In idiopathic infertility studies, the genetic background of patients is favoured (Mallepaly et al., 2017), and when environmental noxious stimuli are considered a cause of reproduction failure, direct exposure *via* different routes is assessed (Ndaw et al., 2018; Upson et al., 2014). At most, gestational exposure to environmental pollutants is considered an indirect burden (Fisher et al., 2020; Monteiro et al., 2020; Ziv-Gal et al., 2015). However, there is limited information regarding breast milk contamination with bisphenols (Tuzimski et al., 2020), as well as the possibly deleterious effects of nursing-mediated exposure in adulthood (Li et al., 2016).

Accordingly, we can consider the nursing period as crucial for testing environmental pollutants due to the following assumed reasons: i) breastfeeding is an exclusive food for infants; ii) pollutants are often liposoluble; iii) therefore, higher concentrations of these compounds may be consumed in fatty-rich breast milk; iv) elimination *via* the glucuronidation metabolic pathway in the infant kidney is not fully developed (Matalová et al., 2016) and, therefore, v) the exposure to low doses may act as a reproductive toxin, despite being harmless to the mother; and finally vi) despite the direct impact of chemical compounds, the metabolic effect of vertical exposure from mother to foetus is considered (summarised in Chemek and Nevoral, 2019).

A multitude of plastic compounds occurs in developed countries, threatening human reproduction. Bisphenols are a widely used plastic

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compound, with endocrine-disrupting properties that ubiquitously affect the endocrine system. Bisphenol A, the most widely used bisphenol compound, is well known for having a negative effect on human reproductive health (Brieno-Enriquez et al., 2012; Mínguez-Alarcón et al., 2015; Peretz et al., 2014) and therefore has gradually been eliminated from many commonly used products (Žalmanová et al., 2016). In particular, its use in baby bottles and toys has been prohibited. Consequently, obvious but regrettable substitutes have been found (Žalmanová et al., 2016): bisphenol S (BPS) and/or BPF that replaced BPA from many consumables, with deleterious BPA-like biological effects (Žalmanová et al., 2017).

While many studies have compared BPA *versus* alternative bisphenols, few studies describing BPA analogues on reproduction have been published in recent years (Desdoits-Lethimonier et al., 2017; Rehfeld et al., 2020; Warner and Flaws, 2018). However, little is known about the molecular mode of the action of bisphenols at relevant doses. Moreover, there is limited information available regarding exposure to BPA analogues, BPS and BPF. Current evidence indicates that gametes are more sensitive to low doses of BPS, whereas the oocyte cytoskeleton, epigenetic code, and/or protein post-translational modifications are affected in a subtle way, depending on the biological impact of bisphenol (Nevoral et al., 2018b; Prokešová et al., 2020; Řimnáčová et al., 2020). These observations appropriately supplement the findings of cohort studies and human biomonitoring, preceding the selection of experimentally used doses. In addition to the direct exposure of adults, gestational transplacental exposure has become a hot topic in human biomonitoring and toxicokinetic studies (Sharma et al., 2018). Similarly, the nursing period has recently come into the centre of interest and is being considered as an exposure window with significant impact on reproduction (Martini et al., 2020). There are several reasons to speculate about this exposure window as a cause of idiopathic infertility induced by environmental pollutants, concurrently being underestimated in the context of reproductive health.

In accordance with the aforementioned evidence, we hypothesised that neonatal exposure to bisphenol analogues leads to female reproductive failure in adulthood, which may otherwise be considered as 'idiopathic'. The aim of the study was to simulate the real-life exposure route to bisphenols being considered safe for reproductive health. Doses have been chosen based on the actual exposure of humankind (Rochester and Bolden, 2015). Moreover, they were used in animal experiments that induced non-fatal systemic responses in exposed animals (Nevoral et al., 2018b; Prokešová et al., 2020). In addition to standard molecular assessment of oocytes, developmental competence of them was evaluated, being considered as a key marker of female fertility (Sirard et al., 2006). Our study provides the first evidence of the biological effect of translactational transfer of alternative bisphenols on female reproduction, using the perinatal nursing window of exposure.

2. Material and methods

All chemicals were purchased from Sigma-Aldrich (MO, USA), unless stated otherwise.

2.1. Animals

Six- to seven-week-old female ICR mice ($n = 60$) were purchased from Velaz Ltd. (Czech Republic) and used as dams of F1 offspring subjected to experimental assessment. The animals were housed in intact polysulphona cages, maintained in 12 h-light/dark cycles at room temperature (21 ± 1 °C) and relative humidity (60%). A phytoestrogen-free diet (1814P; Altromin, Germany) and ultrapure water (in glass bottles, changed twice weekly) were provided *ad libitum*. All animal procedures were conducted in accordance with the Coll. Act on the Protection of Animals against Cruelty No. 246/1992 of the Czech Republic and under the supervision of the Animal Welfare Advisory Committee of the Ministry of Education, Youth, and Sports of the Czech Republic (approval number: MSMT-11925/2016–3).

2.2. Exposure and dosage

ICR dams were mated in the oestrus phase. Bisphenols were administered *via* drinking water (low and moderate BPS, 0.375 ng/mL and 37.5 ng/mL, respectively) during the nursing period of ICR dams between PND0 (*i.e.* day of delivery) and PND15. Vehicle control comprised treatment with 0.1% ethanol in sterile tap water. The exposure duration covered the sensitive exposure window and assured breast milk as an exclusive feeding route for offspring. The route of exposure to bisphenols was selected with respect to the welfare of females exposed during a highly sensitive period of nursing. Doses with known biological effects (Nevoral et al., 2018b) and appropriate to real-life human exposure (Rochester and Bolden, 2015) were chosen. After recording the drinking water consumption and body weight of nursing dams, the actual bisphenol exposure of dams was determined. Henceforward, corrected bisphenol intake was maintained at low and moderate BPS dosages.

2.3. Inspection of clinical signs of reproduction onset

The weight of the litter was inspected at delivery and defined as postnatal day (PND) 0 (PND0). Thereafter, weight was recorded at PND10 and PND21. Once pups were weaned at PND21, anogenital distance, a marker of androgenic activity, was measured as the distance from the superior edge of the external genitalia to the inferior edge of the anus, using a handheld calliper with precision to 0.1 mm. During post-weaning housing, the day of vaginal opening was recorded as the day of onset of puberty.

2.4. Histology and sample preparation

Young and adult ovaries were isolated on PND15 and PND60, when the exposure was terminated and the reproductive peak was achieved, respectively. Ovaries were fixed for 14 days in Bouin's solution, followed by storage in paraffin blocks. Blocks were processed into 10-μm-thick systematic serial sections with random orientations (100 ± 10 sections per animal) using a microtome (Leica RM2255). Every fifth section was mounted on to the slide, followed by staining with the standard protocol with haematoxylin-eosin. From 7 to 14 equidistant sections per ovary, depending on the ovary size, were used for the stereological analysis (Mouton, 2002).

2.5. Stereology and follicle count

Stereological analysis of ovaries was performed using Stereologer 11 software (SRC Biosciences, Tampa, Florida, USA) on a personal computer, attached to a Nikon Eclipse Ti—U microscope, equipped with high-speed XY stage and *Z*-axis motors (Prior, UK), camera (Promicra 3-3 cc), and a standard set of lenses (Plan Fluor). The total volume of the ovaries was estimated, using the point grid counting approach, based on the Cavaslieri principle (Gundersen et al., 1999). The quantification of follicles was performed using optical dissector techniques (Sterio, 1984) by counting visible cell nuclei. The follicles were divided into the following five groups, according to the morphological features described earlier (Nevoral et al., 2018b): primordial, primary, preantral, antral, and atretic follicles. The primordial follicle count was used to estimate the ovarian reserve of young ovaries (PND15) and was excluded from the analysis in adult ovaries (PND60).

2.6. Oocyte isolation

For immature oocytes in the GV stage, we used 8- to 10-week-old perinatally exposed females in the pro-oestrus or oestrus phase. Oocytes were isolated from ovarian tissue using a needle and a 27-G

syringe, and used subsequently for *in-vitro* maturation studies. Alternately, another female was administered PMSG and hCG 48 h later, followed by isolation of *in-vivo*-matured oocytes after 16 h. Ovulated cumulus-oocyte complexes were flushed from the oviduct and treated with 0.1% hyaluronidase for 5 min to remove cumulus cells. These oocytes were used for *in-vitro* fertilisation or immunocytochemistry.

2.7. In-vitro maturation assay

Immature oocytes in the GV stage were manipulated in M2 medium supplemented with 100 μM isobutyl-methylxanthine (IBMX), a specific endogenous phosphodiesterase inhibitor, to maintain intact GV oocytes. Fully grown immature oocytes with intact GVs were placed in M16 culture medium with IBMX and allowed to recover their oocyte pool of proteins for at least 1 h at 37 °C and 5% CO_2. Thereafter, oocytes were fixed in 4% paraformaldehyde in phosphate buffered saline (PBS), supplemented with 0.1% polyvinyl-alcohol, for 30 min at room temperature (22 °C), and stored at 4 °C until further use. Alternatively, recovered GV oocytes were cultured in IBMX-free M16 culture medium for 16 h at 37 °C and 5% CO_2 to obtain matured MII oocytes. Matured oocytes with extruded polar bodies were fixed and stored until further use at 4 °C.

2.8. Immunocytochemistry (ICC) and image analysis

In both *in-vitro*- and *in-vivo*-matured oocytes, α-tubulin and dimethylated histone H3 on lysine K27 (H3K27me2) were used for spindle visualisation and heterochromatin assessment, respectively. Firstly, 4% paraformaldehyde-fixed oocytes were permeabilised in PBS containing 0.04% Triton X-100 and 0.3% Tween-20 for 15 min. Then, oocytes were blocked in 1% bovine serum albumin in PBS with Tween 20 for 15 min and incubated with a cocktail of anti-α-tubulin (1:200, Sigma-Aldrich) and anti-H3K27me2 (1:200, Abcam, UK) primary antibodies. After washing, the oocytes were incubated with a cocktail of anti-mouse and anti-rabbit AlexaFluor 488 and 647 (1:200) antibodies, respectively. Phalloidin (1:200; Thermo Fisher Scientific, USA) was added to the washes and used for β-actin visualisation. Stained oocytes were mounted on to slides in Vectashield medium with DAPI. Signal intensity was measured using ImageJ software. Images were acquired using an Olympus IX83 spinning disc confocal microscope (Olympus, Germany) and VisiView software (Visitron Systems GmbH, Germany). Immunostained oocytes were subjected to measurement of the integrated density (expressing signal intensity) of H3K27me2, using ImageJ software (NIH, Bethesda, CA, USA). The integrated density values were related to control oocytes (VC = 1).

2.9. Parthenogenetic activation

Alternatively to ICC, *in-vivo*-matured oocytes of low and moderate BPS exposure groups were denuded from cumulus cells, as described above, and used for parthenogenetic activation. Oocytes were incubated in modified EmbryoMax KSOM Mouse Embryo (MR-121-D, Millipore) medium, supplemented with 0.1% bovine serum albumin (BSA), 2 mM EGTA, 10 mM $SrCl_2$, and 5 μg/mL cytochalasin B for 5 h at 37 °C and 5% CO_2. Embryos were cultured in KSOM with BSA for 24 h and 96 h to cleaved embryos and blastocyst stages, respectively.

2.10. Statistics

The data were analysed using GraphPad Prism 8 (GraphPad Software Inc., San Diego, CA, USA). Based on Shapiro-Wilks normality distribution tests, differences were tested using an ordinary one-way ANOVA, followed by Tukey's multiple test. Alternatively, Kruskal-Wallis tests and Dunn's post-hoc tests were used for non-normally distributed data. Proportion data were analysed using Fisher's exact test with Bonferoni correction. *P*-values <0.05, 0.01, 0.001, and 0.0001 were considered statistically significant and indicated with asterisks (*), (**), (***), and (****), respectively. Normally and non-normally distributed data were expressed as means and medians, respectively.

3. Results

3.1. Exposure to very low doses of bisphenols does not induce androgenic and/or oestrogenic effects

We aimed to track essential non-invasive characteristics of individual litters, such as weight gain throughout the nursing period, followed by recording of anogenital distance at the day of weaning (defined as the 21st postnatal day, PND21) and the day of vaginal opening following exposure to low and moderate doses of BPS and BPF from delivery to PND15, *via* exposure to doses given to lactating dams. For comparison, two of the most commonly used alternative bisphenols, bisphenol S (BPS) and BPF, were chosen for the initial testing. Diethylstilbestrol (DES), a synthetic nonsteroidal form of oestrogen, was used as a positive control for the potential oestrogen-like effects of low doses of both bisphenols.

Based on the recording of water intake and body weight of nursing dams, the actual bisphenol exposure was calculated (Fig. 1A). There was no effect of perinatal bisphenol exposure on litter weight or weight gain during the nursing period (Fig. 1B). Neither anogenital distance nor vaginal opening, a marker of androgenic and oestrogenic activity, respectively, showed any differences compared to vehicle control (Fig. 1C).

3.2. Ovarian reserve estimation in bisphenol-exposed female pups and adult mice

Histological evaluation of ovarian tissue samples was performed at PND15 and PND60, corresponding to the end of bisphenol treatment and reproductive peak achievement, respectively. No effect of BPS was observed on the primordial follicle reserve in young ovaries (Fig. 2A and Supplementary Table S1). Treatment with low doses of DES did not show any differences. Thus the effect of DES exposure was not evaluated in adult ovaries. Similarly, exposure during lactation to either BPS or BPF did not affect the pool of primary, preantral, and antral follicles in adult ovaries (Fig. 2B and Supplementary Table S2). Even the number of atretic follicles in the low-concentration BPS group was not found as statistically significant ($P = 0.3911$). For complete histological data, see Supplemental Material (Supplementary Tables S1 and S2).

3.3. Assessment of the ovarian reserve of bisphenol-exposed female offspring

Following the findings of histological analyses, the physiological reserve of the ovaries was tested. Females in the proestrus/oestrus phase were used as donors of immature germinal vesicle (GV) oocytes, which are used for *in-vitro* maturation (IVM). Alternatively, hormonally stimulated females ovulated *in-vivo*-matured oocytes.

Based on the number of GV oocytes isolated per ovary, we did not observe any effect of nursing exposure to bisphenols (Fig. 3A). In addition, the ability of meiotic arrest breakdown (GVBD, germinal vesicle breakdown, GVBD) and the maturation stage (maturation rate) were not affected (Fig. 3B). Similarly, the number of flushed *in-vivo*-matured oocytes was not affected, and the number of atretic or fragmented oocytes, indicating oocyte quality, was not altered in bisphenol-exposed offspring (Fig. 3C).

3.4. Bisphenol-affected spindle assembly and histone code in oocytes matured in vitro

Based on the above findings, indicating bisphenol had no effect on oocyte yield or quality, we considered a more tenuous mechanism of

(A)

	bisphenol intake (ng/g bw/day)				
	low BPS (*n*=9)	moderate BPS (*n*=9)	low BPF (*n*=4)	moderate BPF (*n*=5)	low DES (*n*=4)
designed	0.2	20	0.2	20	0.2
actual	0.20±0.01	20.9±0.9	0.23±0.01	25.24±0.4	0.19±0.02

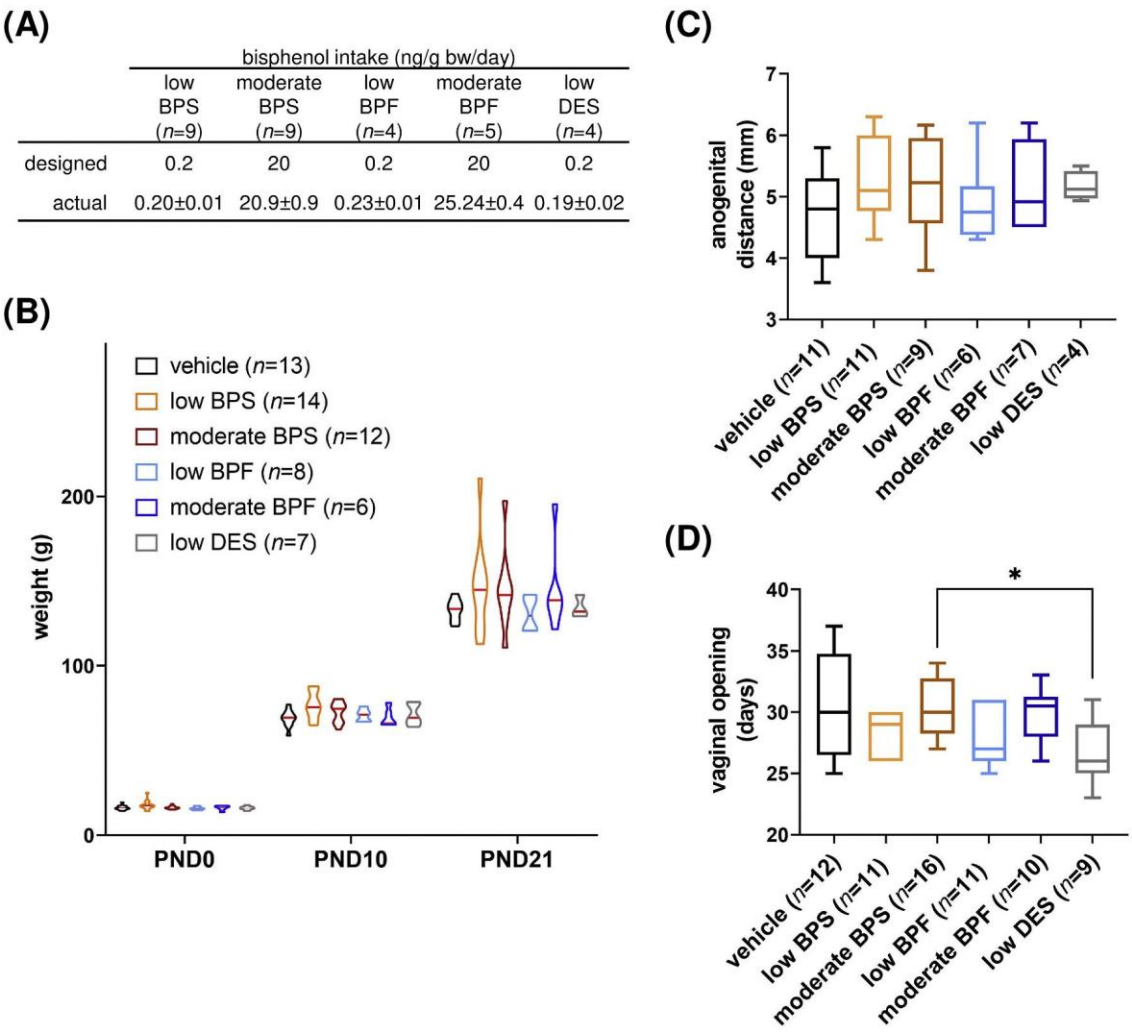

Fig. 1. Non-invasive features of litters and offspring. (A) Planned and effective exposure of dams to bisphenols (BPS, BPF) and diethylstilbestrol (DES), labelled as low and moderate. The number of dams is indicated in brackets. (B) Violin plots showing weight of litters, recorded at birth, *i.e.* postnatal days (PND) PND0, hence after PND10, and PND21. The number of recorded litters is indicated in the legend. The shape of violin plots represents the distribution of individual litters and line points median. (C) Anogenital distance (mm) of female offspring inspected at PND21. (D) The day of vaginal opening. (C,D) The number of inspected female offspring belonging to at least three independent litters is noted in brackets. Data are expressed as medians, and whiskers represent the range of minimum-maximal values. Statistical differences were tested using the Kruskal-Wallis nonparametric test, followed by Dunn's multiple comparisons test. The asterisk indicates statistical significance at $P < 0.05$ (*).

action for low doses of bisphenols at the intracellular level. Immunostaining of α-tubulin and demethylation of histone H3 on lysine K27 (H3K27me2) were chosen as markers of cytoskeleton and histone code-marked heterochromatin, respectively. We considered the native oestrus cycle and selected oocyte donors in the pro−/oestrus phase to avoid any potential interactions with administered gonadotropins.

In-vitro-matured oocytes showed a higher occurrence of spindle abnormalities, accompanied by chromosome misalignment (Fig. 4A). Moreover, persistent astral microtubules were observed in the ooplasm of several oocytes of the low BPS group, along with spindle malformation (Fig. 4B). Indeed, oocytes of female donors exposed to low BPS showed evidence of spindle malformation. Interestingly, only moderate BPF led to a significant increase of spindle misassembly in matured oocytes (Fig. 4C). In addition to abnormalities in ooplasmic microtubules in oocytes exposed to a low level of BPS, a statistically significant increased frequency of chromosomal misalignment was observed (Fig. 4D). A significant decrease in H3K27me2, a chromatin repressive marker, is observed in adult oocytes after a perinatal exposure to either a low dose of BPF or moderate doses of BPS or BPF (Fig. 4E).

3.5. *In-vivo maturation alleviates oocyte damage, but affects developmental competence*

To investigate the effect of nursing exposure on the quality of *in-vitro*-matured oocytes, the latter were subjected to cytoskeletal and histone code assessment of *in-vivo*-matured oocytes of hormonally-stimulated female donors, considered as these oocytes being more resilient to environmental stress (Fig. 5A). There is a significantly increased proportion of oocytes with spindle damage (α-tubulin) and chromosome misalignment in the low BPS group of females (Fig. 5B,C). No changes in histone heterochromatin marker methylation status (H3K27me2) were observed in all bisphenol groups (Fig. 5D).

Following the finding of spindle damage and chromosome misalignment in oocytes of low-BPS-nursing exposed females, *in-vivo*-matured oocytes were used to assess developmental competence. For this purpose, parthenogenetic activation was used, leading to blastocyst formation, while eliminating paternal contribution unlike *in-vitro* fertilisation. Only BPS-affected oocytes were assessed, due to the equality of BPS and BPF phenotypes observed on the oocyte spindle (Fig. 5B). Indeed, the parthenogenetic activation assay indicated a decrease in

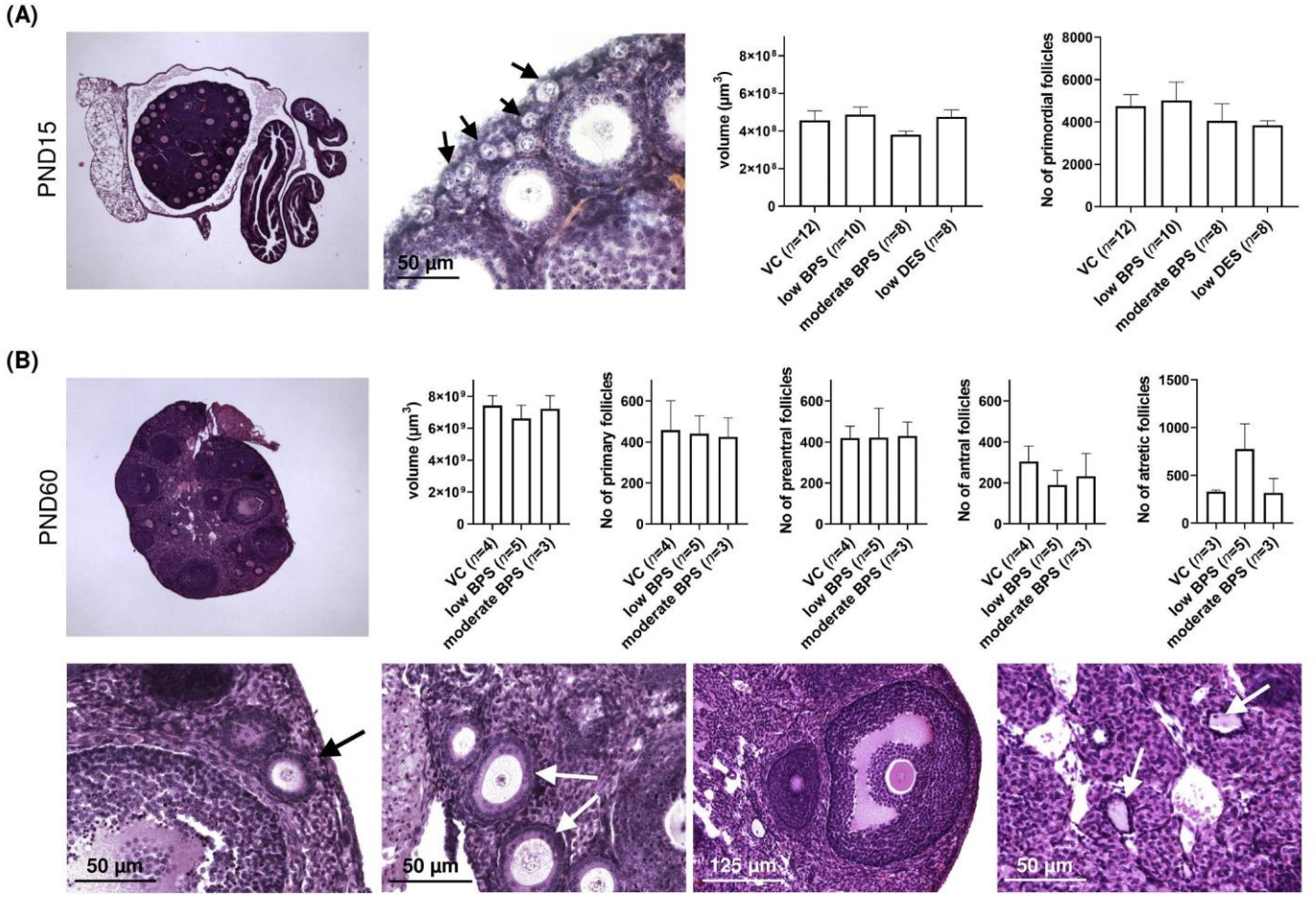

Fig. 2. Histological assessment of young and adult ovaries. (A) Young haematoxylin-eosin-stained ovary on PND15, ovarian volume measurement, and primordial follicle counting. Arrows indicate the primordial follicles assessed. (B) Adult ovary at PND60 and the analysis of ovary volume and the amount of individual follicle stages: primary follicle reserve, preantral, antral, and atretic follicles, including representative pictures of individual stages, respectively. Arrows indicate the type of follicle assessed. The number of female offspring is indicated in brackets. Data had a parametric distribution and are expressed as mean and bars represent standard error of the mean (S.E.M.). Statistical differences were tested using an ordinary one-way ANOVA, followed by Tukey's multiple test.

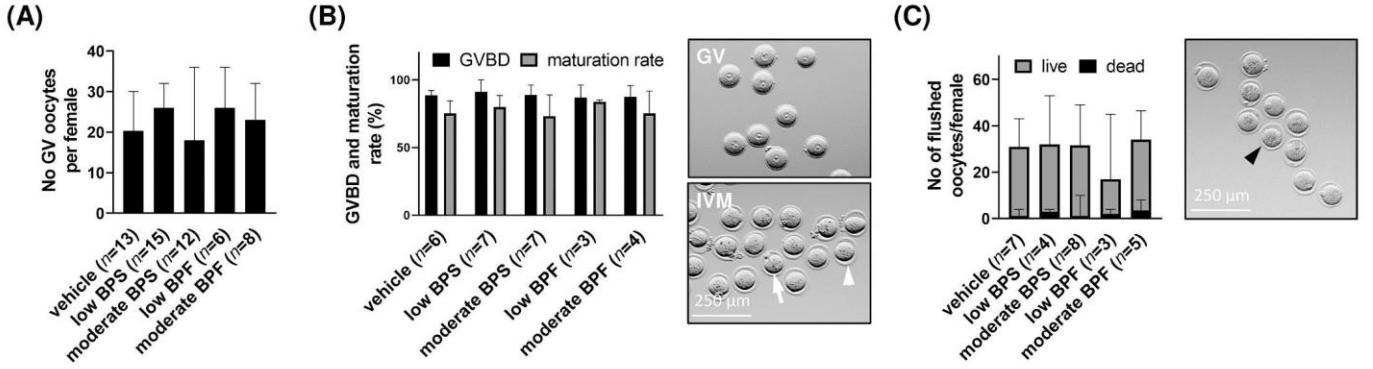

Fig. 3. Effect of perinatal exposure to BPS on female reproductive capacity in adulthood. (A) Follicle capacity expressed by the amount of isolated GV oocytes. The number of female descendent pups is indicated in brackets. (B) Oocyte maturation assessed by the ability to re-initiate meiotic division (GBVD, germinal vesicle breakdown), and to achieve a mature stage, defined as the maturation rate. The number of *in-vitro* maturation (IVM) assays is indicated in parentheses. Arrowhead points to GVBD-underwent oocyte; arrow indicates matured oocytes with extruded polar bodies. (C) The hormonal responsiveness of female descendant due to the counting of number of flushed *in-vivo*- matured and ovulated oocytes. The number of flushed females is indicated in brackets. Arrowhead points to the oocyte classified as dead. All data showed a parametric distribution and they are expressed as mean and bars representing standard error of the mean (S.E.M.). Statistical differences were tested using an ordinary one-way ANOVA, followed by Tukey's multiple test.

developmental competence of ovulated oocytes. The exposure to a low dose of BPS affected the activation and cleavage rate, as well as the blastocyst rate, in contrast to the moderate BPS group (Fig. 5D–E), indicating the association of embryonic development success with the cytoskeletal fitness of matured oocytes.

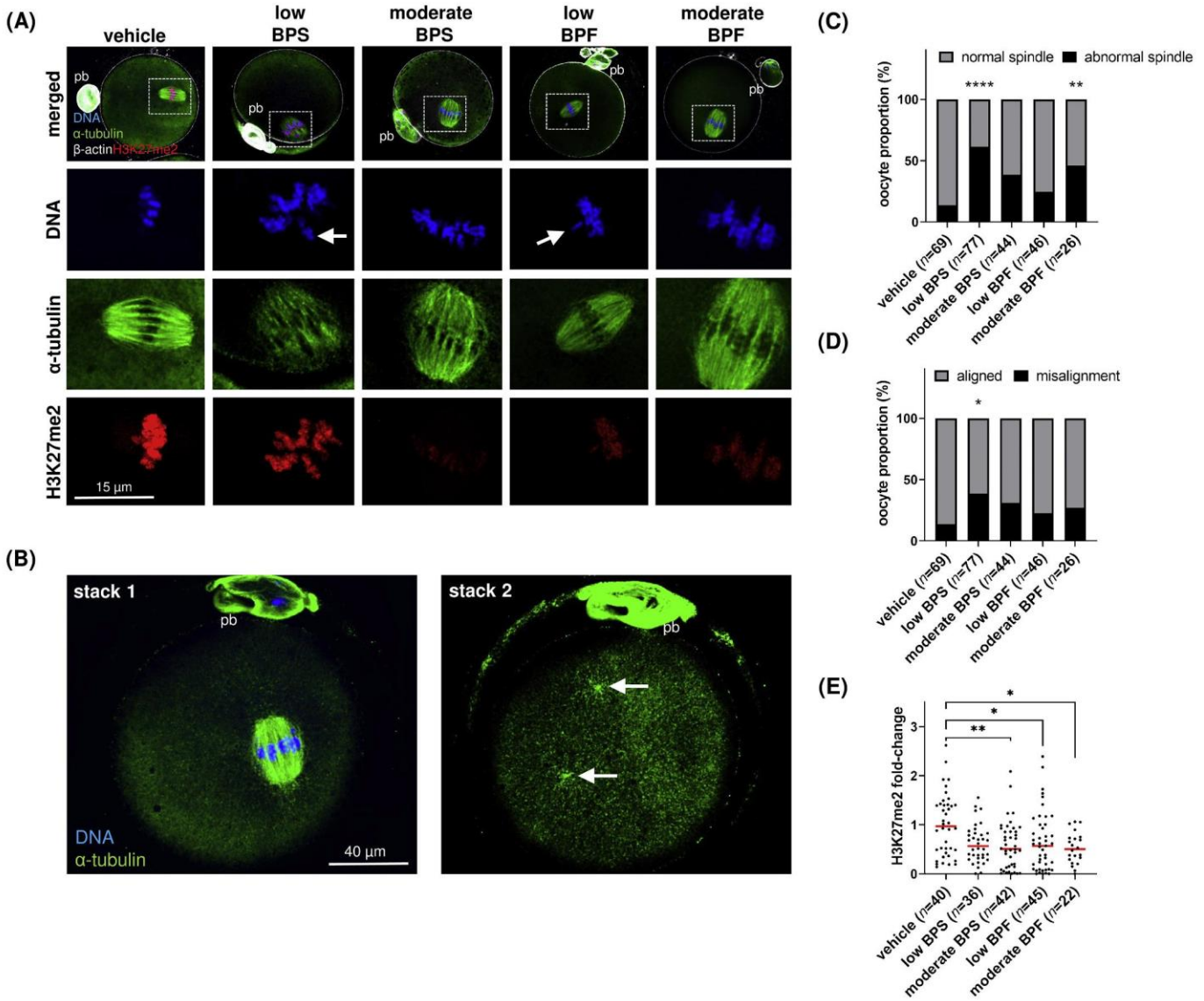

Fig. 4. Effect of nursing bisphenol exposure on cytoskeleton and epigenetic quality of *in-vitro*-matured oocytes. (A) Representative images of chromosome misalignment, cytoskeletal damage of the spindle, and chromosomal H3K27me2. The frame represents the emphasised area of the metaphase chromatin. Arrows indicate chromosome misalignment; pb: polar body. (B) Persisting astral microtubules in the ooplasm (indicated with arrows), accompanying spindle defects of low BPS oocytes. (C) Quantification of matured oocytes carrying the affected spindle, including astral microtubules noted above. (D) Frequency of chromosomal misalignment. (C,D) Data are expressed as cumulative proportion of oocytes, come from at least three unrelated female donors. The numbers of oocytes are indicated in brackets. Statistical differences between control and bisphenol-exposed groups were tested using Fisher's exact test with Bonferoni correction. Asterisks indicate statistical significance at $P < 0.05$ (*), 0.01 (**), and 0.0001 (****). (E) Quantification of H3K27me2 signal density in matured oocytes, related to unexposed control (mean of the vehicle = 1). Data show nonparametric distribution and lines indicating medians. The numbers of analysed oocytes are indicated in brackets. Statistical differences were tested using the Kruskal-Wallis nonparametric test, followed by Dunn's multiple comparisons test. Asterisks indicate statistical significance at $P < 0.05$ (*), and 0.01 (**).

4. Discussion

Investigation into the biological effect of widely used BPA has produced much evidence supporting the deleterious effects of BPA on the endocrine system and on reproduction (Hunt et al., 2003; Moore-Ambriz et al., 2015; Pollock et al., 2014; Rahman et al., 2015; Wang et al., 2016; Ziv-Gal et al., 2015). This has led to a subsequent ban on its use in many commonly used products, such as in polycarbonate plastics and bottles, baby bottles, and toys. For this reason, the BPA analogues, BPS and BPF, have been introduced and used industrially in plastic compounds as a replacement for BPA (Sartain and Hunt, 2016). Recently, several studies have demonstrated the negative effect of these substitutes, and therefore, another 'regrettable substitution' has surfaced (Vandenberg et al., 2012). However, few studies have dealt with very low doses of bisphenols (Jan Nevoral et al., 2018b; Prokešová et al., 2020; Zhang et al., 2020), which mimic actual intake levels *via* environmental exposure in developed countries (summarised by Wu et al., 2018), at most increased in several cases of occupational exposures (Ndaw et al., 2018; Russo et al., 2017; Thayer et al., 2016). In fact, indirect exposure to those low doses through the placenta and/or breast milk are not usually assessed, despite the fact that the exposure windows of *in-utero* development and nursing represent sensitive exposure windows (Dolinoy et al., 2007; Monteiro et al., 2020). Regardless of dosage, the effect of exposure during this exposure window on female reproduction is lacking, although some evidence about translactational exposure to BPA analogues has been published (LaPlante et al., 2017; Li et al., 2016).

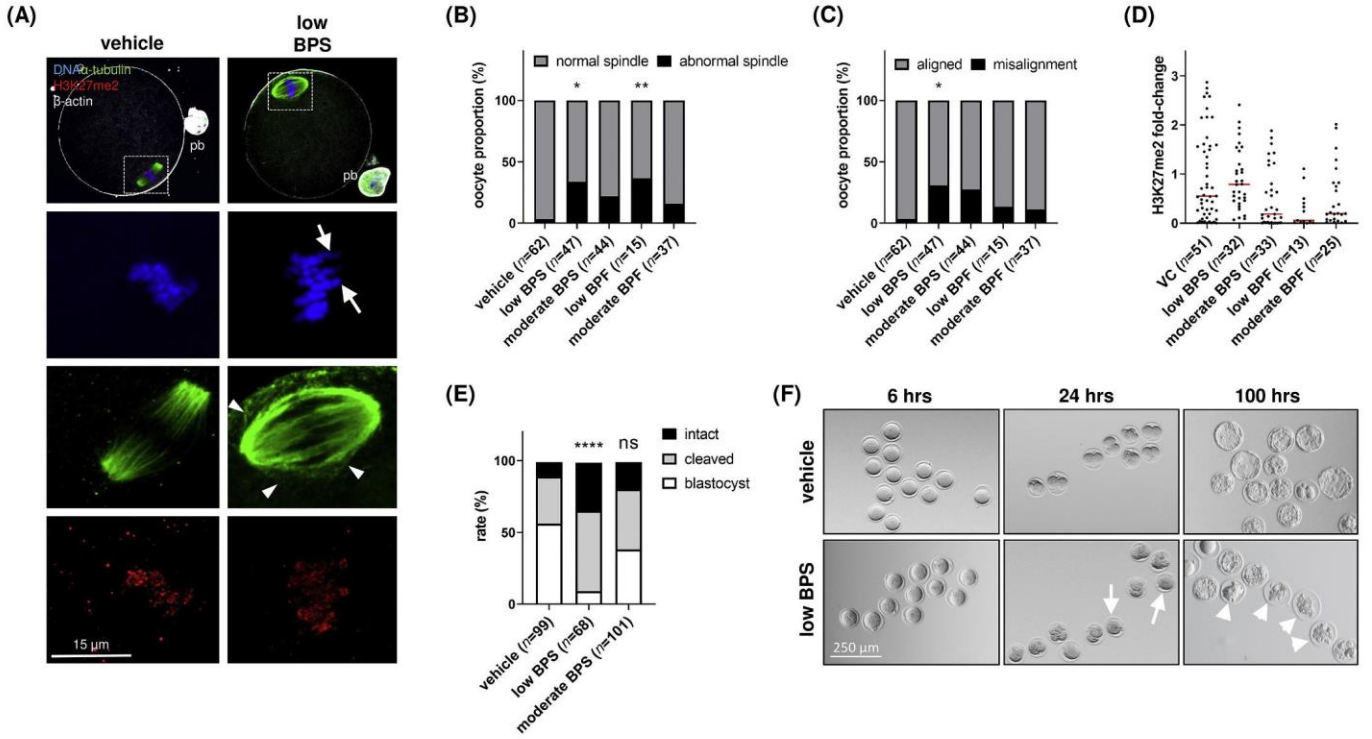

Fig. 5. Effect of nursing bisphenol exposure on the quality of *matured* and ovulated oocytes. (A) Visualisation of oocyte spindles (α-tubulin) and heterochromatin marker H3K27me2. The frame represents the emphasised area of the metaphase chromatin. Arrows indicate chromosome misalignment, arrowheads point to misadjacent tubules on the spindle; pb: polar body. (B) Quantification of matured oocytes carrying the affected spindle. (C) Frequency of chromosomal misalignment. (B,C) Data are expressed as cumulative proportion of oocytes, come from at least three unrelated female donors. The numbers of oocytes are indicated in brackets. Statistical differences between control and bisphenol-exposed groups were tested using Fisher's exact test with Bonferoni correction. Asterisks indicate statistical significance at $P < 0.05$ (*), and 0.01 (**). The numbers of analysed oocytes are indicated in brackets. (D) Quantification of H3K27me2 signal density in matured oocytes, related to unexposed control (mean of the vehicle = 1). Data show nonparametric distribution and lines indicate medians. Statistical differences were tested using the Kruskal-Wallis nonparametric test, followed by Dunn's multiple comparisons test. The number of analysed oocytes is indicated in brackets. (E) The parthenogenetically activated oocytes of BPS-exposed female offspring and the ability of cleavage and blastocyst achievement. Fisher's exact test with Bonferoni correction was used for statistical testing. Asterisks indicate statistical significance at $P < 0.0001$ (****); ns: no significance. The number of activated oocytes is indicated in brackets. (F) Illustrative pictures of activated oocytes of vehicle and low BPS groups, after 6 h, 24 h and 100 h of *in-vitro* embryo culture, leading to pronucleus formation, cleavage, and blastocyst achievement, respectively. Arrows and arrowheads indicate intact non-cleaved oocytes and lysed embryos, respectively.

Furthermore, existing knowledge has been derived mainly from BPA studies using toxic doses (Chen et al., 2020; Qiu et al., 2020), and no specific molecular markers of female fertility have been examined. In this study, we designed a mouse model of translactational exposure to female offspring, through which nursing mothers were exposed orally to bisphenols, to simulate the exposure of nursing mothers to real-life doses of alternative bisphenols, BPS and BPF. Moreover, cytoskeletal and epigenetic markers were analysed in offspring oocytes to uncover the subtle changes in molecular activity of very low doses, in accordance with knowledge of the perinatal period being highly sensitive due to DNA integrity maintenance (Stringer et al., 2020) and epigenetic changes leading to transgenerational inheritance (Pocar et al., 2017, 2012).

In our experiments, we did not observe any obesogenic, androgenic, and/or oestrogenic effects, based on litter weight recording, anogenital distance, and vaginal opening, respectively, in female pups of dams exposed to BPS or BPF. Conventional toxicological approaches cannot detect subtle changes in reproductive functions, leading to 'idiopathic infertility' when suckling offspring transition to adulthood. Therefore, we assumed that there could be impairments identifiable in follicles at different stages of development: i) in young ovaries at PND15, when most oocytes are transcriptionally active and physiologically not bearing meiotic competence and ii) in adult ovaries where follicle recruitment occurs and different stages of follicle development can be found (Sorensen and Wassarman, 1976). In this study, different doses of alternative bisphenols did not induce any significant changes in the number of follicles or in the number of atretic follicles. In accordance with the histological analyses, ovarian reserve was not affected, and the yield of immature and ovulated oocytes was similar for animals exposed to vehicle control and those exposed to both bisphenol doses. Nevertheless, oocyte quality could be affected, while ovarian assessment did not reveal any effects due to bisphenol at low doses.

Our findings showed that the oocyte cytoskeleton was impaired when females were exposed through breast milk to low doses of BPS and BPF, particularly with regard to the meiotic spindles. Moreover, persisting ooplasmic astral microtubules were observed in mature oocytes, seemingly reminiscent of the microtubule-organising centres physiologically occurring in immature oocytes (Verlhac et al., 1993), which persisted in oocytes treated with taxol, a microtubule stabilising agent (Mailhes et al., 1999). These aberrant particles may be considered as non-degraded pericentriolar material or as precursors of centrosomes in oogonia (Sathananthan et al., 2000; Simerly et al., 2018). In addition, spindle resemblance was similar to that observed following excessive polymerisation of tubulin, accompanied by widening of the spindle, and the presence of astral microtubules emanating from spindle poles and/or cytoplasmic foci, found in cryopreserved oocytes (Tamura et al., 2013). Nevertheless, this deviation must not be associated with aneuploidy (Forman et al., 2012). This phenotype is obviously an impact of the exposure on primary oocytes during the perinatal exposure window, while primordial follicles are being formed (Niu and Spradling, 2020).

In addition to spindle assessment, we assumed the epigenetic mode of action of bisphenols. Therefore, H3K27me2 was chosen as a multilateral marker of heterochromatin formation and stability, while its decrease is possibly due to induced apoptosis (Liu et al., 2017) and an obese-mouse oocyte phenotype (Hou et al., 2016). Indeed, *in-vitro*-matured oocytes showed a decrease in H3K27me2 in our experiments, indicating that chromosomal epigenetic assembly repressed heterochromatin formation. Epigenetic remodelling by endocrine disruptors is variable, with the effect of different molecules differing according to the chemical structure. In any case, the epigenetic changes in gametes can potentially lead to the modulation of epigenetic memory and/or shift of gene imprinting, resulting in transgenerational inheritance of these changes (Manikkam et al., 2013; Pocar et al., 2017). Essentially, incorrectly assembled spindles and impaired chromatin stability of *in-vitro*-matured oocytes indicate a defect in the chromosome-segregation machinery in immature oocytes, associated with specific alteration of transcription factors in the ovary of suckling females. Conversely, when the quality of *in-vivo*-matured oocytes was assessed following hormonal stimulation and ovulation of cumulus-oocyte complexes, no significant differences were found.

Our observations of spindle damage and epigenetic remodelling following bisphenol exposure in *in- vitro-* and *in-vivo*-matured oocytes are in accordance with our histological findings, highlighting the significance of an oocyte somatic cell-based environment. Whereas the follicle count was not affected, somatic cell interactions were not able to rescue oocyte quality (Li et al., 2008). This assumption is in accordance with the fact that granulosa cells are differentiated and established in the first days of perinatal life (Niu and Spradling, 2020). Although an *in-vivo* ovulation bottleneck can select oocytes with well-assembled spindles and metaphase plates (Hornak et al., 2012, 2011), we observed spindle damage even in *in-vivo*-ovulated oocytes. Although *in-vivo* oocyte maturation seems to be more robust than *in-vitro* systems, those oocytes can be affected even *in vivo* anyway, leading to the failure of embryonic development. Accordingly, we observed the declined developmental competence of BPS-affected *in-vivo*-matured oocytes using the parthenogenetic activation. Our findings are noticeably similar to earlier published work describing cytoskeletal and epigenetic damage of oocytes following gestational exposure to very low doses of bisphenol S (Zhang et al., 2020).

There are no doubts that further investigation is needed to characterise the targeted proteins. However, several aspects should be taken into consideration: i) developmental competence may be affected by the ability of oocytes to undergo meiotic disruption and maturation; ii) responsible factors are synthesised in young ovaries, seemingly creating a pool not significantly replenished past PND15, which is responsible for the cast-off infertility diagnosed as idiopathic; and iii) surprisingly, the exposure to lower doses of BPS exerts activity which is in contrast to moderate exposure levels of BPS, underlining the non-linear effect of BPS as an agonist of yet unknown targets, further supporting the role of perinatal-induced and bisphenol-driven idiopathic infertility.

5. Conclusions

Alternative bisphenols seem inappropriate and should not be considered as safe BPA substitutes. Both BPS and BPF exert distinct biological effects on oocytes during the perinatal exposure window when the ovarian pool meiotic competence of oocytes is established. Interestingly, individual bisphenols seem to differ in their molecular activity and result in different phenotypes in BPS- *vs.* BPF-exposed females. Further, each BPA substitute is characterised by a different 'effective dose', resulting in different threshold effects. Given the above evidence, bisphenol analogues represent compounds bearing a burden on human reproduction, and through the effects induced during an early exposure window in the breastfeeding infant, these may potentially result in idiopathic infertility in women.

Author contribution

JN designed the study, interpreted, and analysed the data for publication and manuscript preparation. JN, YK, TŽ, JP, and MK contributed to the design of the experiments and critical approval of the final article. JN, JH, YK, ŠP, TF, and LM performed experiments, acquired data, and performed statistical analysis. All co-authors contributed to the manuscript writing.

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Credit author statement

Jan Nevoral: designing of the study, funding acquisition, project administration, conceptualization, methodology, data curation, data analysis and interpretation for publication, manuscript writing (original draft, review, editing) and figures' preparation, formal analysis.

Jiřina Havránková: performing experiments, investigation, visualisation, data analysis (histology), contributing to the manuscript writing.

Yaroslav Kolinko: designing of the study, methodology, interpretation, software, validation, statistics.

Šárka Prokešová: performing of experiments (immunocytochemistry).

Tereza Fenclová: performing of experiments (image analysis).

Ladan Monsef: performed experiments (parthenogenetic activation).

Tereza Žalmanová: designing of the study, writing - review & editing.

Jaroslav Petr: funding acquisition, designing of the study, manuscript writing.

Milena Králíčková: funding acquisition, designing of the study, supervision.

Declaration of Competing Interest

No conflicts of interest are declared.

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Appendix A. Supplementary data

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