ČESKÁ ZEMĚDĚLSKÁ UNIVERZITA V PRAZE

Fakulta agrobiologie, potravinových a přírodních zdrojů

K a t e d r a v e t e r i n á r n í c h d i s c i p l í n

Název disertační práce:

Zvyšování kryoprotektivní účinnosti ředidel semene býků prostřednictvím LDL (Low Density Lipoprotein)

Autor práce: Ing. Ondřej Šimoník

Školitel: Doc. MVDr. Radko Rajmon, Ph.D.

Školitel specialista: Ing. Jiří Šichtař, Ph.D.

Poděkování

Velice rád bych zde poděkoval mému školiteli **Doc. MVDr. Radko Rajmonovi, Ph.D.**

Radko, moc Ti děkuji vůbec za možnost stát se členem Katedry veterinárních disciplín. Dále Ti děkuji za čas, který si věnoval čtení a korekcím textů, kterými jsem Tě celá ta dlouhá léta zásoboval. Rovněž Ti děkuji za předávání zkušeností pedagogických a praktických.

Další velké díky putuje **Ing. Jiřímu Šichtařovi, Ph.D.**

Jirko, moc díky za tvé rady a velkou podporu v těžkých situacích, ty si mi to vždycky uměl vysvětlit…. Děkuji Ti za cenné rady, předání vědeckých zkušeností a tvůj čas strávený v laboratoři a při psaní publikací.

Do třetice chci vyjádřit své díky **RNDr. Pavle Postlerové, Ph.D.**

Pavli, veliké díky za tvou ochotu, vstřícnost a předání cenných vědeckých zkušeností. Děkuji za možnost pracovat po tvém boku v laboratořích i při psaní publikací, bylo a je to velice inspirativní.

Poděkování patří také **Prof. Joaquínu Gadeovi** a **Prof. Carmen Matás** jedinečným osobnostem a vynikajícím vědcům za jejich cenné rady praktické zkušenosti a za možnost být na čas součástí Grupo de Fisiología de la Reproducción na Veterinární univerzitě v Murcii.

Zvláštní poděkování patří mé nejbližší rodině, kterou se v průběhu mého doktorského studia stala partnerka **Lenka Tůmová** a následně dcerka **Kačenka**.

Leni děkuji za nesmírnou podporu, vždy si mi ukázala cestu. Děkuji Ti za odborné rady, cenné připomínky a rodinnou pohodu. Děkuji Ti Kačenko za tvou toleranci mého nočního ťukání do klávesnice při psaní této práce.

Samozřejmě moje velké díky patří mým rodičům a prarodičům, za jejich neutuchající podporu od začátků mých studií. **Rodičové**, **babi** a **dědo** – DĚKUJI!

Čestné prohlášení

Prohlašuji, že disertační práci "Zvyšování kryoprotektivní účinnosti ředidel semene býků prostřednictvím LDL (Low Density Lipoprotein)" jsem vypracoval samostatně pod vedením vedoucího práce a s použitím odborné literatury, které jsou citovány v práci a uvedeny v seznamu literatury na konci práce. Jako autor uvedené disertační práce dále prohlašuji, že jsem v souvislosti s jejím vytvořením neporušil autorská práva třetích osob.

V Praze dne

Obsah

1 Úvod

Umělá inseminace je biotechnologická metoda, která je nejvíce využívána v chovu mléčného skotu z důvodu její snadné proveditelnosti, úspěšnosti a ekonomické návratnosti (Aires et al., 2003; Moore et Hasler, 2017). Jsou používány především kryokonzervované inseminační dávky (ID). Poslední statistické údaje uvádějí celosvětovou produkci 252 miliony hluboce zmrazených ID ročně a 130 milionů provedených inseminací (Thibier et Wagner, 2002; Moore et Hasler, 2017). Právě kryokonzervace zajišťující kompletní zastavení fyziologických pochodů a vývojových procesů spermií (Vishwanath, 2003) je jedním z hlavních faktorů, které ovlivnily rozšíření UI do celosvětového měřítka. Významnou výhodou UI je rozšíření možností využitelnosti geneticky vhodných a prověřených jedinců, s čímž souvisí mnohonásobně vyšší využití ejakulátu býka v porovnání s přirozenou plemenitbou (Whiston et al., 2017; Woods et al., 2004). Dále je to prakticky neomezená vzdálenost transportu ID a zamezení šíření venerologických onemocnění (Bailey et al., 2003). I přes všechny výhody UI je však faktem, že významné procento spermií je v průběhu procesu kryokonzervace poškozeno (Watson, 2000; Srivastava et al., 2015; Layek et al., 2016).

Prvním z objevů, týkajících se prodloužení životnosti spermií po odběru ejakulátu, bylo odhalení pozitivních účinků vaječného žloutku. Hlavním milníkem v souvislosti přímo s kryokonzervací spermií byl náhodný objev kryoprotektivních vlastností účinků glycerolu při experimentech se spermiemi býků a narození prvního telete po UI takto uchovávanými spermiemi (Polge et Rowson, 1952). Od tohoto objevu se optimalizací procesu kryokonzervace spermií býků zabývalo mnoho dalších výzkumných prací. I přesto však není v dnešní době procento přeživších, oplození schopných spermií uspokojivé (Layek et al., 2016). Z tohoto důvodu je důležité zkoumat možnosti ovlivňování efektivity procesu kryokonzervace. Jednou z hlavních cest je optimalizace a modifikace složení ředidel semene (Watson, 2000; Hu et al., 2010; Yeste, 2015), a tím redukce negativních vlivů působících na spermie v průběhu celého procesu kryokonzervace.

Zvyšování efektivity a optimalizace procesu kryokonzervace spermií je tedy důležité jak pro zvyšování úspěšnosti umělé inseminace skotu, tak z hlediska přínosu nových poznatků do vědního oboru kryobiologie.

2 Literární přehled

2.1 Faktory ovlivňující spermie v průběhu procesu kryokonzervace

I přes dlouhé období, jež uběhlo od zásadního objevu kryoprotektivních vlastností glycerolu a úspěšného zmrazení spermií, byly protokoly vypracované na počátku padesátých let jen mírně pozměněny. Všeobecně se uvádí, že oplozovací schopnost hluboce zmrazených ID je dostačující (Hu et al., 2010). Faktem ovšem zůstává, že je stále 40–50 % spermií během tohoto procesu nenávratně poškozeno (Layek et al., 2016). V průběhu procesu kryokonzervace jsou spermie vystaveny podmínkám vymykajícím se fyziologickým standardům. Je zde řada fyzikálních a chemických stresových faktorů ovlivňujících jejich strukturální a funkční integritu (Morris et al., 2012). Do výčtu těchto vlivů jsou řazeny: teplotní změny, tvorba intracelulárních a extracelulárních ledových krystalů, odchylky extracelulárního prostředí od izotonických podmínek spojené s toxickým působením kryoprotektantů a oxidativní stres (Gadea et al., 2008; Tapia et al., 2012).

2.1.1 Teplotní změny

Spermie savců nejsou adaptovány k přežití teplot, kterých je dosahováno v průběhu procesu kryokonzervace, a to především z hlediska lipidového složení a propustnosti iontů plazmatickou membránou. To je kompenzováno možností transformace struktury lipidů v plazmatické membráně (PM) odehrávající se při teplotách od 36 do 17 °C (Parks et Graham, 1992; Sparr et al., 2002), přičemž k největším změnám ve struktuře lipidů dochází při teplotách 15–5°C (Drobnis et al., 1993). Tyto změny jsou popisovány jako tzv. chladový šok. Na tento stresový faktor reagují spermie rozdílně, v závislosti na druhu, což je dáno především odlišným lipidovým složením PM a různou mírou citlivosti spermií jednotlivých druhů zvířat ke kryokonzervaci (Srivastava et al., 2013). Jeho letální následky lze částečně zredukovat vhodnou rychlostí poklesu teplot při zchlazování spermií (Benson et al., 2012). Tento fenomén ovlivňuje další komponenty PM, jako jsou struktury proteinového charakteru, které se mohou vlivem alterací lipidů shlukovat, a může docházet k narušení jejich funkčních vlastností. Takovým příkladem mohou být iontové kanály CatSper (Chen et al., 2017). Jedním z hlavních faktorů poukazujících na průběh chladového šoku je tak narušení permeability PM a její regulace. Jak uvádí Drobnis et al. (1993), dochází ke snižování koncentrace intracelulárního K^+ a zvyšování Ca^{2+}, což následně ovlivňuje aktivitu bičíku (motilitu) a další struktury spermií. Důsledkem zvýšené koncentrace vápenatých kationtů mohou být také předčasné kapacitační změny, které negativně ovlivňují potenciální fertilizační schopnost spermií (Srivastava et al., 2015).

2.1.2 Tvorba intracelulárních krystalů

Další faktor ovlivňující přežitelnost spermií při procesu kryokonzervace je tvorba ledových krystalů. Zde platí všeobecně známé pravidlo říkající, že struktura buněk zmrazených příliš rychle je nenávratně poškozena kvůli tvorbě intracelulárních krystalů vody a v opačném případě buňky zchlazované příliš pomalu jsou poškozovány dlouhou expozicí prostředí o vysoké koncentraci solutů (Benson et al., 2012). Redukce či v některých případech eliminace těchto negativních vlivů je docíleno využíváním vhodných kryoprotektantů a mrazících křivek (Pena et al., 2011). V nedávné době bylo však kolektivem autorů (Morris et al., 2007) pomocí elektronové mikroskopie a diferenciální skenovací kalorimetrie prokázáno, že při současně využívaných mrazících křivkách pro spermie hřebců již nedochází k tvorbě intracelulárních krystalů. Potenciální nebezpečí poškození buněk tak proto představují především krystaly extracelulární.

2.1.3 Tvorba extracelulárních krystalů

S tvorbou extracelulárních ledových krystalů nepřímo souvisí další ze stresových faktorů negativně ovlivňující funkční vlastnosti spermií změny v osmotických podmínkách. Tento typ krystalů vystavuje spermie hypertonickým podmínkám (Koshimoto et al., 2000), a to díky procesu nukleace ledových krystalů potažmo vytvoření krystalů vody při zchlazení média k teplotě tuhnutí. Soluty jsou poté disociovány ve zbývající dosud nezmrzlé vodě, čímž stoupá osmotický tlak roztoku, v němž jsou spermie přítomny (Watson, 2000).

Jak dále upřesňuje Holt (2000b) a je rovněž patrné z výsledků elektronové mikroskopie (Morris et al., 1999), spermie individuálně mohou být ve zmrzlém médiu současně lokalizovány v podmínkách s vyšší či nižší koncentrací solí. Pro omezení negativního vlivu extracelulárních krystalů platí všeobecné pravidlo, že při vyšší rychlosti zchlazování suspenze (spermie + ředidlo) dochází k omezení přítomnosti dosud nezmrzlé vody a tím ke snížení působení hyperosmotických podmínek (Benson et al., 2012). Zároveň však musí být rychlost zmrazování dostatečně pomalá ve vztahu k eliminaci letálních krystalů intracelulárních (Woods et al., 2004; Benson et al., 2012).

2.1.4 Přítomnost permeabilních kryoprotektantů

K odchylkám od izotonických podmínek přispívá nezanedbatelným dílem rovněž přítomnost permeabilních kryoprotektantů v ředidlech (Tapia et al., 2012).

Přidání ředidla k ejakulátu má tak významný dopad na tvorbu hypertonického charakteru extracelulárního prostředí. Intracelulární voda je tak ve směru nižšího chemického potenciálu

(vyšší koncentrace osmoticky aktivních látek) transportována ven z buněk a paralelně dochází ke zmenšování objemu spermií (Mazur et Koshimoto, 2002). V průběhu rozmrazování je situace opačná, spermie jsou vystavovány podmínkám hypoosmotickým. V důsledku tohoto je voda do cytoplasmy přijímána až do té doby, než dojde k vyrovnání koncentrací solutů mezi intracelulárním a extracelulárním prostorem, tím je tedy jejich objem zvětšován. Právě kvůli těmto dvojnásobně působícím osmotickým změnám, na které jsou spermie velmi citlivé, dochází primárně k porušení integrity plazmatické membrány (Tapia et al., 2012). Navíc bylo zjištěno u spermií býků (Guthrie et al., 2002) a hřebců (Macias Garcia et al., 2011c), že fluktuace v osmotických podmínkách působí více negativně v porovnání s konstantním vystavováním hypertonickým podmínkám.

2.1.5 Oxidativní stres

Oxidativním stresem se rozumí stav nerovnováhy mezi produkcí reaktivních forem kyslíku a antioxidativním systémem sloužícím k jejich redukci či k regeneraci struktur, které již byly zasaženy. Právě zmiňované reaktivní formy kyslíku (Reactive Oxygen Species - ROS) jsou hlavními sloučeninami působícími negativní změny (Agarwal et al., 2003). Jestliže je tvorba ROS v rovnovážném stavu s funkcí antioxidativního systému, působí na fyziologické procesy spermií např. na kapacitaci a akrozomální reakci (O'Flaherty et Matsushita-Fournier, 2017). ROS jsou sloučeniny vytvářené biochemickými reakcemi mezi inertním kyslíkem a organickými molekulami. Zahrnují především volné radikály, což jsou chemické látky, které obsahují minimálně jeden nepárový elektron a jsou přitom stabilní. Tento fakt zajišťuje právě jejich vysokou reaktivitu s organickými sloučeninami (Griveau et LeLannou, 1997).

Gamety jsou všeobecně velmi citlivé právě na změny způsobené ROS. V případě spermií je tato citlivost způsobena specifickým složením plazmatické membrány, omezenými schopnostmi antioxidativního systému a neschopností opětovné syntézy poškozených komponent (Gadea et al., 2013). V průběhu procesu kryokonzervace jsou spermie vystavovány zvýšeným koncentracím těchto vysoce reaktivních sloučenin, přičemž s jejich zvýšenou produkcí souvisí změny v průběhu chladového šoku (Chatterjee et al., 2001). Toxicita ROS souvisí s inaktivací proteinů, poškozením DNA a peroxidací nenasycených lipidů (O'Flaherty et Matsushita-Fournier, 2017). Poslední uvedený vliv je v případě spermií velmi významný z hlediska vysokého podílu fosfolipidů v plazmatické membráně obsahujících řetězce polynenasycených kyselin. To ve výsledku vede k destabilizaci plazmatické membrány, a tím k modifikaci aktivity enzymů na ni vázaných a nakonec k buněčné smrti (Aitken et Baker, 2004). Mimo tento uvedený vliv má kryokonzervace negativní dopad na koncentraci intracelulárního

glutationu, který hraje velice významnou roli v antioxidativním systému spermií býků i dalších druhů (Gadea et al., 2011; Castro et al., 2016). Souhrnem těchto vlivů je tedy ve výsledku opět potenciálně snižována schopnost spermií přežít a zachovat si oplozovací schopnost.

Výše uvedené faktory negativně ovlivňují spermie v průběhu procesu kryokonzervace. Přičemž primární strukturou, na kterou má kryokonzervace především dopad, je plazmatická membrána (Khalil et al., 2018).

2.2 Plazmatická membrána – kritická struktura z pohledu kryokonzervace

Plazmatická membrána pokrývající celou spermii je typickým příkladem vysoce kompartmentalizované struktury. V průběhu spermatogeneze, při diferenciaci kulatých spermatid ve spermie protáhlého tvaru, je PM dělena na 3 kompartmenty, jenž tvoří tzv. subdomény mající specifický komplement proteinů a lipidů (Gadella et Luna, 2014). První z nich pokrývající hlavičku spermie jsou součástí akrozómu, ekvatoriálního segmentu a postakrozomální části. Další dva kompartmenty se nachází na střední části v hlavním oddíle bičíku (Gadella et al., 1994). Mezi těmito jednotlivými subdoménami dochází k výměně membránových komponent a jsou tak zajišťovány fyziologické procesy a jejich kontrola (James et al., 2004). Tzv. kompartmentalizace PM je z hlediska kryokonzervace vlastností, která může působit negativně, a to kvůli skutečnosti, že dává jednotlivým částem různé vlastnosti (Morris et al., 2012).

2.2.1 Složení plazmatické membrány spermií

Buněčná membrána je tvořena lipidovou dvojvrstvou tvořenou zejména fosfolipidy skládajícími se z polární a nepolární části interagujících s řadou proteinů. Orientace polárních částí (hlaviček) lipidů, jež mohou mít různou chemickou strukturu, je směrem k vodnému prostředí. Nepolární části jsou tedy orientovány směrem k sobě (Tapia et al., 2012). Distribuce lipidů ve dvojvrstvě je nehomogenní, neustále dochází k laterálním změnám jejich pozic. Tento pohyb též nazývaný „flip-flop“ je umožňován činností ATP dependentních enzymů flipáz a flopáz. Posledním typem enzymů, který je však na ATP nezávislý, jsou skramblázy, fungující opačně tzn. jejich funkcí je omezení asymetrie lipidové dvojvrstvy PM.

Složení PM spermií je specifické, jsou zde glycerol-fosfolipidy s převahou fosfatydilcholinu a fosfatydiletanolaminu, jejichž zastoupení je mezidruhově stálé (Parks et Lynch, 1992). Dále jsou zde fosfatydilserin, fosfatydilinositol, lysofosfatydilcholin, sfingomyelin, kardiolipin (Gadella et Luna, 2014). Glycerol-fosfolipidy, všeobecně, jsou

charakteristické svou stavbou, základní strukturou je glycerol, na kterém jsou hydroxylové skupiny na C1, C2 esterifikovány v mastné kyseliny a skupina OH- na C3 je esterifikována na fosfát. Významná část těchto fosfolipidů je však složena z plazmalogenů, v nichž jsou dlouhé nenasycené řetězce mastných kyselin (C-20; C-24) vázány přes vazbu éterovou (Lenzi et al., 2000; Le Guillou et al., 2013). Spermie se od ostatních typů savčích buněk dále výrazně liší obsahem plasmalogenů, které v některých případech představují více jak 18 % z celkového množství glycerol-fosfolipidů tvořících jejich PM (Oresti et al., 2011). Jak uvádí Fuchs et al. (2007) velmi pravděpodobně mají schopnost eliminovat volné radikály a dále je zde předpoklad, že jsou důležité pro tvorbu makro a mikrodomén potřebných z hlediska kompartmentalizace PM spermií (Gorgas et al., 2006).

Z hlediska kryokonzervace jsou významné mezidruhové rozdíly ve složení PM, především z hlediska zastoupení mastných kyselin, přesněji jejich zbytků tvořících hydrofilní konce fosfolipidů a jejich stupně nasycenosti (Tapia et al., 2012). Nejvýznamněji jsou ve struktuře PM spermií hospodářských zvířat zastoupeny acylové zbytky kyseliny dokosahexanové (C22:6 n-3) a dokosapentaenové (22:5 n-6). U spermií býků je nejčastěji zastoupenou mastnou kyselinou ve struktuře fosfolipidů C22:6 n-3 (Schiller et al., 2003), stejně tak u spermií kanců (Cerolini et al., 2000), u hřebců je to 22:5 n-6 (Macias Garcia et al., 2011b). Vysoký obsah zbytků polynenasycených mastných kyselin dává plazmatické membráně spermií výraznou fluiditu, která zajišťuje vyšší elasticitu. Přičemž tato vlastnost přímo ovlivňuje schopnost buňky přizpůsobovat svůj objem daným podmínkám (Wathes et al., 2007). Všeobecně lze říci, že vyšší podíl polynenasycených mastných kyselin v PM pozitivně koreluje s procentem spermií vykazujícím intaktní PM, právě z důvodu zvýšení fluidity, potažmo flexibility PM. Opačný vliv má pak vyšší podíl dlouhých nasycených řetězců mastných kyselin, jenž způsobuje nepoddajnost PM (Macias Garcia et al., 2011b). Na druhou stranu však zvýšený výskyt násobných vazeb ve struktuře acylových zbytků znamená vyšší citlivost k poškození volnými radikály, a tudíž náchylnost k peroxidaci lipidů PM (Wathes et al., 2007). Úroveň zastoupení určitých mastných kyselin v PM hraje významnou roli především z hlediska senzitivity spermií vůči kryokonzervaci v rámci mezidruhových i individuálních rozdílů (Macias Garcia et al., 2011b).

Dalším velice důležitým ukazatelem udávajícím senzitivitu PM ke kryokonzervaci je poměr cholesterolu k fosfolipidům. Cholesterol je podstatnou složkou PM, představuje 25–50 % lipidů PM, což tvoří až 90 % celkového buněčného cholesterolu. Strukturálně se steroly liší od ostatních polárních lipidů tím, že jejich hydrofilní část je tvořena malými hydroxylovými skupinami. Ty jsou navázány na hydrofobní část tvořenou čtyřmi cyklickými uhlíkatými řetězci,

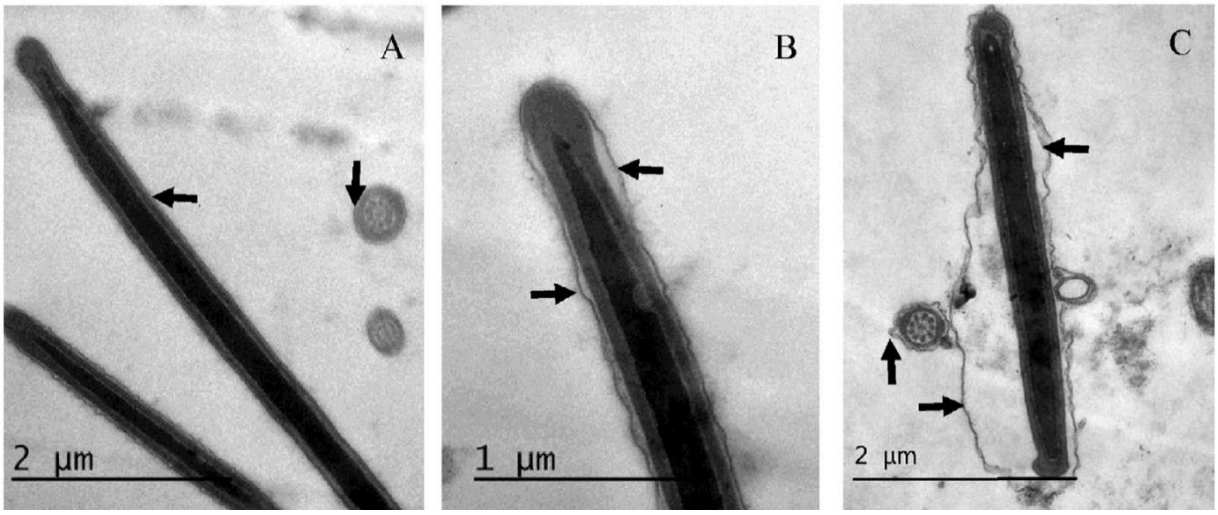
kde je na opačném konci než se nachází skupina OH-, připojen rozvětvený uhlíkatý řetězec (Moce et al., 2010a). Cholesterol je důležitým prvkem regulace membránové fluidity. Nad hranicí teploty ovlivňující strukturální podobu lipidů PM ji snižuje a naopak při teplotách pod touto hranicí ji zvyšuje (Purdy et Graham, 2004). Bylo potvrzeno, že změna lipidového složení PM týkající se zvýšení procentuálního zastoupení cholesterolu, vlivem přídavku cyklodextrinů nesoucí tuto sloučeninu, představuje pro spermie vyšší odolnost vůči chladovému šoku a přežitelnost po kryokonzervaci (Purdy et Graham, 2004; Moce et al., 2010b). Zvýšený podíl cholesterolu by mohl rovněž snížit změny podobné kapacitaci spermií vyvolávané kryokonzervací, které negativně ovlivňují jejich fertilizační schopnosti (Cormier et Bailey, 2003). Ze sterolů je dále zastoupen desmosterol. Jeho přítomnost byla objevena již v roce 1974 autory Bleau et Vandenhe, avšak prozatím nebyl zjištěn jeho přesný význam. Předpokládá se, že jeho úloha bude obdobná, jako byla potvrzena v případě cholesterolu (Travis et Kopf, 2002; Saez et al., 2011).

Zatímco fosfolipidy představují strukturální elementy PM, membránové proteiny plní její specifické funkce. Proteiny jsou děleny do dvou základních skupin podle jejich připojení na PM a to na transmembránové a periferní. Proteiny transmembránové jsou začleněny přímo do lipidové dvojvrstvy. Především jako iontové kanály a přenašeče se účastní selektivního přenosu molekul skrz membránu a dále regulačních a signálních procesů. Druhou skupinu tvoří periferní proteiny, jež jsou na PM nepřímo navázány pomocí interakce s polárními hlavičkami fosfolipidů a zaujímají hlavní roli při oplození a regulaci dalších fyziologických procesů (Tapia et al., 2012). Reorganizací lipidů v průběhu kryokonzervace a narušením jejich interakce s proteinovou složkou může rovněž docházet k jejímu nevratnému poškození. To může způsobit změny vedoucí k fenoménu všeobecně nazývanému „kryokapacitace“ (Cormier et Bailey, 2003), jelikož je PM důležitým prvkem v signální kaskádě procesu kapacitace spermií (Signorelli et al., 2012).

2.2.2 Změny v plazmatické membráně v průběhu kryokonzervace

Plazmatická membrána je za fyziologických podmínek charakteristická svým fluidním stavem. Fluidita je jednou ze základních vlastností PM a je ovlivňována především poměrem obsahu fosfolipidů a cholesterolu a také teplotou. Při poklesu teploty na určitou mez dochází ke změnám v uspořádání lipidů v PM. Při zchlazování se lipidy transformují, což vede ke změně fluidního stavu PM v přechodný - na rozhraní fluidní a krystalické formy. Současně dochází ke změnám v uspořádání acylových zbytků (Oldenhof et al., 2015; Sieme et al., 2015). Poté je v průběhu zmrazování měněn přechodný stav PM v krystalický, kdy se současně s pokračující změnou postavení zbytků mastných kyselin stává PM rigidní. Teplota, při které dochází k těmto změnám, se liší v závislosti na délce uhlovodíkových řetězců a jejich nasycenosti. Jakmile je této teploty dosaženo, dojde ke změně konformace daného typu lipidů, které poté agregují s ostatními lipidy v PM nacházejícími se ve stejném stavu. Část lipidů však zůstává za těchto podmínek ve fluidním stavu až do dosažení následující specifické teplotní změny. Právě přechody mezi částmi PM, kde již přechod lipidů do krystalické formy proběhl a částmi ve fluidním stavu, představují slabá místa, kde může dojít k mechanickému poškození (Hammerstedt et al., 1990; Sieme et al., 2015). K poškození až do tohoto stádia nemusí vždy dojít, narušení PM může být na úrovni funkce iontových kanálů a dalších regulačních částí. Tato populace spermií může být pak v částečně kapacitovaném stavu, což znamená jejich zkrácenou životnost a sníženou fertilizační schopnost (Bailey et al., 2000; Bailey, 2010). Z hlediska procesu kryokonzervace, je to obsah cholesterolu v PM, jenž udává její rezistenci vůči nízkým teplotám. Hraje roli při stabilizaci PM (Bergeron et al., 2003; Aksoy et al., 2010), i přes to, že je fluidita PM v přítomnosti většího množství cholesterolu vyšší, si spermie zachovávají výrazně lepší viabilitu (Purdy et Graham, 2004). Cholesterol působí také pozitivně při stabilizaci řízení aktivity transmembránových proteinů (Tannert et al., 2007). Spermie s vyšším podílem cholesterolu k fosfolipidům jsou charakteristické vyšší odolností vůči procesu kryokonzervace (Moce et al., 2010b).

Změny způsobené zmiňovanými faktory mají v průběhu kryokonzervace negativní dopad primárně na plazmatickou membránu spermií (Yeste, 2015). Ultrastrukturální studie Khalil et al. (2018) tento fakt na základě výsledků elektronové mikroskopie jasně potvrdila (viz. Obr. 1). Její integrita však dále souvisí s dalšími strukturami a organelami, jako je akrozóm či mitochondrie (Sieme et al., 2015; Khalil et al., 2018). Přičemž narušení jejich morfologické a funkční integrity (Morris et al., 2012) následně vede ke snížení jejich fertilizační schopnosti (Oldenhof et al., 2015). Odolnost PM spermií je dána především lipidovým složením, zejména co se týče přítomnosti cholesterolu společně se zastoupením polynenasycených mastných kyselin (Macias Garcia et al., 2011a; Yeste, 2015).



Obr. 1 Změny na PM býčích spermií v průběhu procesu kryokonzervace. **A** ihned po odběru; **B** v průběhu zchlazování; **C** po kryokonzervaci (Khalil et al., 2018)

Právě opatření vedoucí k potlačení změn na plazmatické membráně či k modifikaci jejího složení představují možnosti pro zdokonalování kryokonzervačních protokolů především s důrazem na složení ředidel semene (Hu et al., 2011; Yeste, 2015).

2.3 Ředidla používaná pro kryokonzervaci semene býků

Jelikož spermie čelí během procesu kryokonzervace řadě negativně působících vlivů, musí být pro kryokonzervaci spermií použito vhodné ředidlo. Všeobecně musí být v receptuře ředidel semene přítomny složky (iontové nebo neiontové povahy) udržující osmolaritu a pH. Dále je důležitý zdroj lipoproteinů či jiných vysokomolekulárních složek, jako vaječný žloutek či mléko, působící jako nepermeabilní kryoprotektanty proti chladovému šoku (Holt, 2000a; Layek et al., 2016). Další složkou je glycerol či alternativy jako propan-diol, dimethylsulfoxid (DMSO), acetamid či etylen-glykol představující permeabilní kryoprotektanty. Především prvně

jmenovaný glycerol je v největší míře používán v ředidlech pro kryokonzervaci spermií býků (Vishwanath et Shannon, 2000; Morris et al., 2012). Pro tyto permeabilní kryoprotektanty je z hlediska jejich použití významná hranice jejich toxicity (Fahy, 2010). Jako zdroj energie slouží v receptuře ředidel glukóza, fruktóza a jiné jednoduché cukry. Navíc mohou některé sacharidy, jako trehalóza či rafinóza, ve vyšších koncentracích mít podobný kryoprotektivní účinek, jako je tomu u permeabilních kryoprotektantů (Malo et al., 2010). Poslední složkou ředidel semene jsou aditiva, jako enzymy, antibiotika pro minimalizaci růstu mikroorganismů původem ze semenné plazmy a zamezení kontaminace. Dále to mohou být aminokyseliny ochraňující strukturu plazmatické membrány nebo také antioxidanty (Gadea et al., 2011; Moore et Hasler, 2017).

Rozdělení druhů ředidel je dáno především na základě přítomného zdroje vysokomolekulárních kryoprotektivních sloučenin nepermeabilního charakteru (Layek et al., 2016). Jsou to ředidla s těmito složkami výhradně neživočišného původu, a to v největší míře s obsahem extraktu ze sóji – sójového lecitinu (fosfatydilcholinu) např. Biociphos®, Bioxcell® (IMV, Francie), Andromed® (Minitübe, Německo). Dalším druhem jsou ředidla s obsahem vaječného žloutku, např. BullXcell (IMV, Francie), Triladyl® (Minitübe, Německo), Optidyl® (Biovet, Francie) nebo na bázi odstředěného mléka např. Laciphos® (IMV, Francie). Právě ředidla, v nichž je obsažen vaječný žloutek, jsou hojně využívaná při kryokonzervaci spermií býků a dalších hospodářských zvířat (Leite et al., 2010; Singh et al., 2014; Layek et al., 2016). Pozitivní vlastnosti ředidel na bázi vaječného žloutku jsou však negativně ovlivňovány několika faktory. V první řadě je to skutečnost, že ne všechny složky vaječného žloutku jsou charakteristické pozitivním působením v průběhu kryokonzervace. Některé složky, jako vysokodenzitní lipoprotein (High Density Lipoprotein - HDL) obsažený ve žloutkových granulách, mohou negativně působit na respiraci spermií (Demianowicz et Strzezek, 1996; Wall et Foote, 1999). Navíc vaječný žloutek představuje možné hygienické riziko, že mikrobiální kontaminace ředidla s obsahem žloutku je srovnatelná s kontaminací samotného žloutku (Bousseau et al., 1998; Aires et al., 2003). K této skutečnosti se přidává další faktor, kterým je variabilita složení vaječného žloutku, což přímo souvisí s problémem standardizace receptury ředidla (Aires et al., 2003; Singh et al., 2014). Dále může přítomnost žloutku negativně ovlivnit laboratorní postupy při mikroskopickém hodnocení parametrů spermií (van Wagtendonk-de Leeuw et al., 2000; El-Sharawy et al., 2012).

Potenciální překážky a rizika ředidel na bázi vaječného žloutku byly omezeny uvedením ředidel s obsahem sójového lecitinu (Layek et al., 2016). Nebyl však prokázán srovnatelný vliv této kryoprotektivní složky. Dosavadní studie hodnotící kryoprotektivní schopnosti ředidel na bázi sójového lecitinu a vaječného žloutku ve vztahu k *in vitro* a *in vivo* ukazatelům prokázaly různorodé výsledky, ve větší míře ve prospěch ředidel s obsahem vaječného žloutku (Gil et al., 2000; van Wagtendonk-de Leeuw et al., 2000; Thun et al., 2002; Aires et al., 2003; Crespilho et al., 2012).

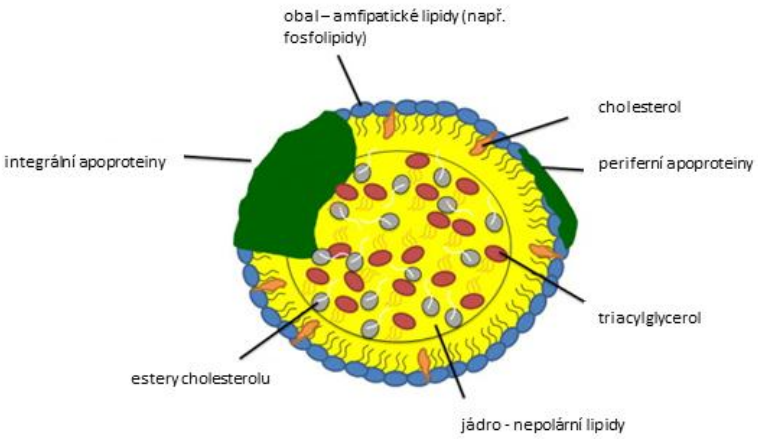
Po vyvinutí receptury ředidel s obsahem sójového lecitinu následoval další důležitý krok spojený s modifikací kryokonzervace spermií. Byl zdokonalen postup získávání kryoprotektivní složky vaječného žloutku – LDL a byly testovány jeho kryoprotektivní účinky (Moussa et al., 2002; Anton et al., 2003), což otevřelo prostor pro další možnosti modifikace složení médií pro kryokonzervaci spermií.

2.4 LDL kryoprotektivní složka vaječného žloutku

Další možností jak předcházet negativním vlivům vaječného žloutku je tedy využití pouze jeho funkční, kryoprotektivní složky – LDL. Vaječný žloutek obsahuje 78 % plazmy a 22 % granul. Jde o velice komplexní směs lipoproteinů a proteinů, u nichž dosud není známa jejich přesná funkce. Žloutková granula obsahují hlavně lipoproteiny o vysoké hustotě, 70 % (High Density Lipoprotein), a 16 % fosfitinů (Hevonoja et al., 2000). Naproti tomu žloutková plazma obsahuje především nízkodenzitní lipoprotein (Low Density Lipoprotein – LDL), který zahrnuje polovinu obsažených fosfolipidů a cholesterol, jež působí jako ochrana proti chladovému šoku (Murphy et al., 2017). Žloutková plazma s majoritním zastoupením LDL je tedy zodpovědná za kryoprotektivní vlastnosti žloutku (Pace et Graham, 1974; Moussa et al., 2002).

2.4.1 Charakteristika LDL a jeho kryoprotektivní působení

LDL tvoří dvě třetiny hmoty vaječného žloutku, jsou to sférické částice o velikosti 17–60 nm. Lipidové jádro je složeno z triglyceridů a esterů cholesterolu, obklopováno vrstvou fosfolipidů a proteinů (viz. Obr. 2). Celkově obsahuje 83–89 % lipidů a 11–17 % proteinů (Moussa et al., 2002; Anton et al., 2003). LDL bylo separováno poprvé již v roce 1974 (Pace et Graham, 1974) následně byla metoda separace zdokonalena ve smyslu vyšší efektivity produkce standardizace a čistoty frakce LDL (Moussa et al., 2002).



Obr. 2 Ilustrativní nákres struktury částice LDL (Harisa et Alanazi, 2014)

Přesné mechanismy kryoprotektivního působení LDL nejsou dosud přesně popsány. Jedním z možných mechanismů je schopnost LDL přilnout k PM spermií a vytvořit tak ochranný film (Bergeron et al., 2004). Bylo zjištěno, že LDL hraje významnou roli v průběhu procesu gelace vaječného žloutku (Wakamatu et al., 1982; Primacella et al., 2018). Při tomto procesu, který je spouštěn kryokonzervací, dochází k rozvolnění struktury LDL. Vazby mezi lipidy a fosfolipidy jsou narušovány, přičemž právě naopak je tomu u vazeb mezi proteiny. Triglyceridy a fosfolipidy jsou tak uvolňovány do média a apoproteiny obalující jádro částic LDL se stávají méně rozpustnými a vytváří gel. Fosfolipidy poté mohou tvořit ochranný film na povrchu PM (Hu et al., 2010). Je však také možné, že by mohly být celé molekuly vázány na PM spermií (Bergeron et al., 2004). Těmito interakcemi může být vytvářena mechanická bariéra chránící fázové rozhraní PM proti narušení (Martinet et al., 2003). Navíc dochází k inkorporaci LDL do struktury PM, a tím ke zvyšování obsahu cholesterolu a fosfolipidů v plazmatické membráně, což znamená jejich vyšší odolnost v průběhu kryokonzervace (Bergeron et Manjunath, 2006). Dalším mechanismem působení LDL na spermie je jeho schopnost vytvářet velice stabilní komplexy s BSP-A1, BSP-A2, BSP-A3 a BSP-30-kDa, souhrnně nazývané BSP (Bovine Sperm Binder) proteiny (Lusignan et al., 2011b). Ty tvoří 80 % celkového množství bílkovin semenné plazmy. BSP jsou na fosfatydilcholin v PM spermií vázány ihned po ejakulaci (Srivastava et al., 2012), dále mají schopnost vázat se na kapacitační faktory (heparin, HDL), z čehož vyplývá jejich pozitivní efekt. Avšak na druhou stranu je zde také jiný efekt než ten fyziologický. Jsou-li spermie vystavovány proteinům po delší dobu, jako např. v průběhu procesu kryokonzervace, je jejich účinek negativní kvůli schopnosti těchto proteinů stimulovat vylučování cholesterolu a fosfolipidů z PM (Lusignan et al., 2011b; Rodriguez-Martinez et al., 2011; Srivastava et al., 2013). To vede ke zvýšení citlivosti spermií na proces kryokonzervace ve vztahu např.

k chladovému šoku (Therien et al., 1999; Morris et al., 2012) nebo vlivem destabilizace PM může dojít k předčasným kapacitačním změnám spermií (Cormier et Bailey, 2003; Faezah et al., 2014). LDL, jak bylo zjištěno autory (Lusignan et al., 2011b), je charakteristické velkou kapacitou pro vazbu těchto proteinů, a navíc vytvořené vazby jsou velice stálé, a to i po rozmrazení. Dále bylo prokázáno, že vazba LDL na BSP proteiny je nasycená, rychlá a specifická (Manjunath et al., 2002; Lusignan et al., 2011b). Jiným pozitivním vlivem LDL je jeho příznivé působení na antioxidativní aktivitu enzymů katalázy (CAT), glutation peroxidázy (GSH-Px) a redukovaného glutationu (GSH), které hrají důležitou roli při zachování fertilizační schopnosti spermií (Hu et al., 2011). Kvůli vysokému podílu polynenasycených mastných kyselin v plazmatické membráně, jsou spermie citlivé právě na poškození oxidativním stresem, jenž je vysokou měrou ovlivňován kryokonzervací (Tapia et al., 2012). Právě antioxidativní aktivita výše jmenovaných enzymů byla díky přítomnosti LDL v ředidle prokazatelně zvýšena (Hu et al., 2011; Perumal et al., 2016).

Výše uvedené mechanismy působení LDL příznivě ovlivňují kvalitativní parametry spermií a působí tak pozitivně na udržení jejich fertilizační schopnosti po rozmrazení. Veškeré studie zabývající se efektem LDL na spermie býků po rozmrazení hodnotily jeho vliv v podobě náhrady vaječného žloutku v receptuře ředidel. Autoři Moussa et al. (2002) kromě zdokonalení postupu získávání LDL, jako první odhalili skutečnost, že optimální koncentrací je 8 % LDL v ředidle z hlediska procentuální motility spermií býků po rozmrazení. Dalšími autory byl tento fakt potvrzen a doplněn o další výsledky poukazující na pozitivní vliv této koncentrace LDL na další *in vitro* parametry spermií, jako je integrita plazmatické a akrozomální membrány zachování aktivity mitochondrií či integrity DNA (Amirat et al., 2005; Hu et al., 2010; Hu et al., 2011).

Pouze v omezeném počtu studií zabývajících se efektem LDL na spermie býků byly zahrnuty ředidla na bázi sójového lecitinu a to pouze v kontrolních skupinách. Jak z hlediska motility spermií, tak integrity plazmatické a akrozomální membrány byly tyto parametry signifikantně zlepšeny vlivem LDL, které v ředidlech nahrazovalo vaječný žloutek, v porovnání s ředidly s obsahem rostlinného lecitinu (Amirat et al., 2005; Vera-Munoz et al., 2011).

Ve všech výše uvedených studiích nebyl popsán žádný postup konzervace LDL po jeho získání. Z toho vyplývá menší úroveň jeho potenciálního praktického využití. To by mohlo být zdokonaleno možností konzervace LDL pomocí azidu sodného, který by mohl mimo prodloužení doby jeho použitelnosti rovněž eliminovat také hygienická rizika (Hyldgaard et al.,

2014), jež jsou hojně diskutovaným tématem (Bousseau et al., 1998; Aires et al., 2003; Layek et al.; 2016).

Absence konzervace LDL tak otevírá otázku možnosti otestování jeho ošetření po výrobě pomocí azidu sodného. To by znamenalo jeho stabilizaci, omezení mikrobiálního rizika, prodloužení doby použitelnosti a tím zvýšení potenciální úspěšné translace výsledků výzkumu do praxe.

Pozitivní efekty LDL byly ve všech případech testovány a potvrzeny v souvislosti s eliminací vaječného žloutku z receptury ředidel, tedy jeho substituce pomocí LDL (např. Moussa et al., 2002; Hu et al., 2011). Jen v omezeném počtu studií byly pro srovnání dynamických vlastností ředidel zařazeny i vzorky spermií, v kontrolních skupinách, ošetřené ředidly na bázi sójového lecitinu. I v tomto případě spermie ředěné v ředidlech, kde byl vaječný žloutek nahrazen LDL vykazovaly po rozmrazení lepší kvalitativní parametry v porovnání s ředidly s obsahem sójového lecitinu (Amirat et al., 2004; Amirat et al., 2005; Amirat-Briand et al., 2009).

Dosavadní studie zabývající se srovnáním kryoprotektivního efektu ředidel s obsahem vaječného žloutku s ředidly na bázi sójového lecitinu neprokázaly shodující se výsledky. S ohledem na potvrzené pozitivní efekty LDL vyvstává tedy otázka, zda by se pozitivní účinky LDL mohly projevit ve spojení s ředidly na bázi sójového lecitinu a dojde tak ke zlepšení jejich kryoprotektivních vlastností. Navíc ve prospěch této hypotézy hraje roli i skutečnost, že LDL je možno využívat jako bio-nanomolekulu při transportu terapeutických substancí na buněčné úrovni (Harisa et Alanazi, 2014), což ukazuje na jeho vynikající schopnost interakce s PM.

2.5 Hodnocení kvalitativních parametrů spermií

Hodnocení kvalitativních ukazatelů spermií slouží k posouzení jejich funkčního stavu související do jisté míry s jejich potenciální fertilizační schopností. Na inseminačních stanicích je z praktického hlediska prováděno hodnocení omezeného počtu parametrů spermií, a to v rámci vstupní kvality ejakulátu po odběru a následně pro ověření úspěšnosti kryokonzervace. Ve výzkumu, při vývoji nových postupů týkající se zpracování spermií, kam patří samozřejmě i modifikace ředidel semene, je k hodnocení jejího vlivu využívána širší škála funkčních parametrů spermií.

Jedním ze základních ukazatelů kvality spermií je jejich motilita (Sellem et al., 2015). Toto hodnocení může být prováděno dvěma způsoby. Subjektivní hodnocení s sebou nese řadu

možných vlivů, které mohou výsledek zatížit chybou (Contri et al., 2010). Významně objektivnější metodou s větší možností standardizace v rámci určité laboratoře je počítačem řízená analýza spermií (Computer Assisted Sperm Analysis - CASA) (Amann et Waberski, 2014). V tomto případě nehraje hlavní roli lidské oko, ale kamera převádějící pohyb spermií do digitální podoby a počítačový software se specifickými algoritmy, který vypočítává řadu individuálních kinematických parametrů společně s ukazatelem procentuální celkové a progresivní motility. Avšak i v případě použití této metody může dojít k zatížení výsledků chybou a to ve všech fázích procesu hodnocení vzorku od použití vhodné komůrky po vhodné statistické zpracování a interpretaci dat (Holt et al., 2007; Gallego et al., 2017). Tato metoda byla využívána v našich studiích, bylo tedy velmi relevantní se hlouběji zabývat jak faktory s možným dopadem na kvalitu výsledků, tak i dalším vývojem stávajícího softwaru v laboratoři Katedry veterinárních disciplin. Z tohoto důvodu bylo naplánováno zpracování review zaměřeného na aktuální stav poznání týkající se CASA s důrazem na faktory ovlivňující výsledky a metody zpracování dat. Pro zpřesněním výsledků kvalitativní analýzy spermií po rozmrazení bylo nutné doplnit výše uvedené hodnocení motility o analýzu dalších funkčních parametrů (Sellem et al., 2015). Mezi ty patří v první řadě viabilita, primárně spojována s integritou plazmatické membrány (Gillan et al., 2008). Intaktnost této struktury může být hodnocena na základě schopnosti buňky nepřijmout do cytoplasmy barvivo Eosin. Sofistikovanější alternativou pro tuto základní metodu je použití fluorochromů charakteristických jejich vyšší specifitou. Jde například o karboxyfluorescein di-acetát nebo SYBR 14 v kombinaci s propidium jodidem, který je nejčastěji využíván pro značení mrtvých buněk z důvodu schopnosti interkalace mezi porušené báze DNA (Kumar et al., 2017). SYBR 14 je jedna z nejlepších variant díky jedinečné vlastnosti, být vázán pouze na neporušenou DNA, proto podává velice objektivní výsledky i při hodnocení spermií po rozmrazení, kde je velké riziko interference fluorochromu se složkami ředidla (Graham et al., 1990; Petrunkina et al., 2010). Další strukturou nezbytnou pro úspěšné oplození je akrozóm. Jeho integritu je možné fluorescenčně stanovit nejčastěji na základě specifické vazby lektinů na sacharidy obsažené v akrozómu. Přičemž pro hodnocení spermií býků je uváděno vhodnější použití lektinu původem z *Pisum Sativum* (PSA). Soubor funkčních parametrů, které v současnosti již patří na experimentální úrovni ke standardům, uzavírá stanovení mitochondriální aktivity. Nejčastěji je využíváno fluorochromu MitoTracker, kdy princip funkce spočívá v jeho akumulaci uvnitř funkčních mitochondriích. Přičemž musí být bráno na zřetel, zda bude využita aldehydová fixace či nikoli.

U těchto fluorescenčních metod je nespornou výhodou vysoká specifita a možnost využití průtokové cytometrie přinášející vysokou objektivitu výsledků z důvodu velkého počtu pozorovaných spermií v porovnání s počítáním buněk na mikroskopických preparátech (Hossain et al., 2011).

3 Hypotézy a cíle

Byly stanoveny hypotézy:

- Modifikace receptury vybraných ředidel pomocí LDL bude mít pozitivní dopad na rezistenci spermií vůči chladovému šoku
- Modifikace receptury vybraných ředidel na bázi sójového lecitinu pomocí LDL povede ke zvýšení jejich kryoprotektivních vlastností
- Substitucí vaječného žloutku v ředidle pomocí LDL ošetřeného azidem sodným bude dosaženo výsledků srovnatelných se standardy uváděnými v literatuře.

Pro ověření hypotéz byly stanoveny následující cíle:

- Stanovit efekt LDL v ředidlech semene na rezistenci spermií býků vůči chladovému šoku před kryokonzervací
- Stanovit efekt adice LDL do ředidel na bázi sójového lecitinu na funkční parametry spermií býků po kryokonzervaci
- Ověřit standardní účinnost LDL při substituci vaječného žloutku v ředidle v porovnání s publikovanými standardy s ohledem na použití azidu sodného pro jeho dlouhodobé uchovávání
- Optimalizovat metodiku pro hodnocení motility spermií pomocí počítačem řízené analýzy (CASA)
    - Analýza aktuálního stavu problematiky
    - Aplikace teoretických a praktických poznatků do praxe v laboratoři Katedry veterinárních disciplin včetně modifikace stávajícího softwaru.

4 Publikované práce

4.1 Počítačem řízená analýza spermií současný stav poznání, faktory ovlivňující výsledky a translace poznatků do modulu CASA - NIS Elements 4.50

Motilita spermií je jeden ze základních ukazatelů funkční integrity potažmo fertility po jejich kryokonzervaci. V posledních letech se zvýšila objektivita metod hodnocení díky zavedení počítačem řízené analýzy spermií (anglický ekvivalent Computer Assisted Sperm Analysis - CASA) (Mortimer, 2000; Amann et Waberski, 2014). Z výzkumné sféry se v poslední době značnou měrou rozšířila do praxe a je hojně využívána pro kontrolu kvality na inseminačních stanicích býků (Kathiravan et al., 2011). Pro dosažení zmiňované vyšší objektivity a opakovatelnosti výsledků je nutné dodržet řadu zásad a doporučení, které poté vedou jak k vyšší porovnatelnosti výsledků napříč laboratořemi, tak ke zpřesnění tolik žádané predikce fertility spermií následně v podmínkách *in vivo*.

V této souvislosti byl stanoven cíl: Optimalizovat metodiku pro hodnocení motility spermií pomocí počítačem řízené analýzy (CASA).

Na Katedře veterinárních disciplin je dlouhodobě používán software NIS Elements (Laboratory Imaging s.r.o., Praha). Ovšem, co se týče modulu CASA, musel být z hlediska specifických požadavků modifikován. Z tohoto důvodu bylo zpracováno podrobné review, které se soustředilo na informace o klíčových aspektech této metody shrnující aktuální poznatky týkající se celé řady faktorů ovlivňujících výstupy a metodické postupy zpracování dat. Dále to byly i praktické zkušenosti z několikaměsíční stáže na Veterinární fakultě Univerzity v Murcii (Španělsko), jež umožnily aktivní podíl na dalším vývoji modulu CASA.
V rámci spolupráce s touto firmou byl tak modul na základě připomínek a návrhů modifikován a byla vytvořena jeho finální podoba. Co se týče konkrétních změn v softwaru, byl především doplněn o důležité funkce zajišťující zpřesnění výsledků, kontrolu při analýze nasnímaných vzorků a uživatelsky příjemnější práci se získanými daty.

Bylo tak dosaženo translace teoretických a praktických poznatků do praxe laboratoře Katedry veterinárních disciplin. Konkrétně šlo o tyto nejdůležitější softwarové modifikace: I)

přidání/odebrání nasnímaných objektů z následující analýzy; II) kontrolní výpočet koncentrace před snímáním spermií; III) okamžitý výpočet celkové a progresivní procentuální motility spermií; IV) automatický export dat do souboru Excel; V) uživatelské rozhraní zajišťující lepší kontrolu nad pořízenými daty.

Tímto byly vytvořeny podmínky pro experimentální studie týkající se tématu této disertační práce a rovněž pro další výzkumné týmy Katedry veterinárních disciplin.

Publikace:

Simonik, O., Sichtar, J., Krejcarkova, A., Rajmon, R., Stadnik, L., Beran, J., Dolezalová, M., Biniova, Z. 2015. Computer assisted sperm analysis – the relationship to bull field fertility, possible errors and their impact on outputs: A review. Indian Journal of Animal Sciences. 85 (1). 3-11.

Dalším výstupem na základě zpracovaného review a praktických zkušeností ze zahraničních stáží bylo vytvoření detailního manuálu pro práci s modulem CASA softwaru NIS Elements 4.50. (viz příloha č 1.)

Indian Journal of Animal Sciences **85** (1): 3–11, January 2015/Article

Computer assisted sperm analysis – the relationship to bull field fertility, possible errors and their impact on outputs: A review

O SIMONIK[1], J SICHTAR[2], A KREJCARKOVA[3], R RAJMON[4], L STADNIK[5], J BERAN[6], M DOLEZALOVA[7] and Z BINIOVA[8]

Czech University of Life Sciences Prague, Kamycka 129, 165 21 Praha 6 – Suchdol, Czech Republic

Received: 28 March 2014; Accepted 23 September 2014

ABSTRACT

Sperm motility is one of the indicators most evaluated before and after cryopreservation, regarding quality and fertilizing ability. The present review provides complex information about the possible negative effects on the results of computer assisted sperm analysis (CASA) and also reflects a possible connection of these results to bull field fertility. Recently, there has been a growing interest in sperm motility assessment by CASA to determine sperm motion more accurately and objectively than by subjective evaluation. CASA systems have been routinely used in most research laboratories and also with increasing tendency in the case of insemination centres. However, objectivity and comparison of CASA results through laboratories can be impacted unfavourably. This is in particular due to the absence of standardization for bull sperm motility evaluation and the presence of drawbacks in the form of human and non-human factors. Investigators have recently turned to the possible association of CASA results with the prediction of bull field fertility. However, the studies suffer from discrepancies, thus a clear relationship has not yet been confirmed. Specific combinations of motility parameters with accurate determination of sperm subpopulations could represent another part in the complex system of providing the ability to predict fertility *in vivo*. The task of future works should be to establish standardization regarding sperm motility evaluation of specific animals, in addition to the settings and algorithms of CASA systems. Furthermore, predictive value CASA outputs to bull field fertility demand more extensive research aimed at a more precise definition of this relationship.

Key words: CASA, Fertility, Insemination dose, Sperm motility

For successful fertilization, both the male and the female gamete of every species is important. In males, this factor is currently represented by the quality of insemination dose (Beran *et al.* 2012, Beran *et al.* 2013) mainly in cattle, where artificial insemination (AI) is the biotechnological method used on a wide scale (Muino *et al.* 2008, Gravance *et al.* 2009, Sundararaman *et al.* 2012).

Quality control of insemination doses before and after thawing is mostly carried out subjectively, mainly on the basis of an estimation of motile spermatozoa ratio, since sperm motility is considered as closely related to fertility (Puglisi *et al.* 2012). Results of this estimation can be affected by bias and inaccuracy (Amann and Waberski 2014); moreover there are discrepancies among results of different studies (Farrell *et al.* 1998, Januskauskas *et al.* 1999, Gillan *et al.* 2008, Kathiravan *et al.* 2008). Methods routinely used for the assessment of spermatozoa do not always give results correlating sufficiently with fertility (Puglisi *et al.* 2012) and also these outputs are laden by subjectivity and variability (Rijsselaere *et al.* 2012).

In comparison to subjective evaluation, during examination of sperm suspension with computer assisted sperm analysis (CASA), a greater number of sperm cells is semi-automatically evaluated and, moreover, in a shorter time (Verstegen *et al.* 2002, Kathiravan *et al.* 2011). CASA represents a practical tool, which was developed at the beginning of the 1980s, aimed at providing a more objective analysis of sperm motility by reconstruction of sperm trajectories and their classification into different categories, to provide further details of sperm motility for quality assessment and fertility estimation. Nevertheless, there are still difficulties which have to be resolved before the routine use of CASA, as well as requirements which have to be fulfilled and factors which can affect CASA results (Mortimer 1997, Feitsma *et al.* 2011). According to Kathiravan *et al.* (2011), this system of sperm motility analysis can be considered as a more effective, precise and trustworthy tool for fertility assessment than subjective

Present address:[1,3,7,8]Ph.D. student (simoniko@af.czu.cz, krejcarkova@af.czu.cz, dolezalovamartina@af.czu.cz, zuzana@naturalgen.cz), [2]Post-doc (sichtar@af.czu.cz), [5]Associated Professor (stadnik@af.czu.cz).[6]Scientific worker (beranj@af.czu.cz), Czech University of life Sciences, Department of Animal Husbandry. [4]Associated Professor (rajmon@af.czu.cz), Head of Department, Veterinary Sciences, CULS in Prague.

evaluation across breeds and age categories of breeding males. They also state that the results obtained are more objective, repeatable with a higher predictive ability and can be standardized. However, there are requirements which have to be fulfilled and factors which influence the results.

A primary disadvantage of CASA is the fairly high price of these systems which relates to its availability for laboratories. An overwhelming majority of managers and laboratory leaders have extensive information from CASA suppliers, reassuring them of accurate and reliable results. However, independent accurate comparison among different CASA systems has been lacking. Moreover, there is no standard among different CASA types (Kathiravan *et al.* 2011, Lu *et al.* 2014). Despite the indicated results by suppliers, it is appropriate to carry out comparison among different CASA systems and performance tests before purchasing (Feitsma *et al.* 2011, Amann and Waberski 2014). According to Feitsma *et al.* (2011), it is recommended that tests are performed on at least 100 ejaculates in duplicates, with the intention of evaluating accuracy and repeatability. The repeatability of the CASA results can be determined by regression of CASA measurement and WHO standards. The coefficient of determination resulting from this analysis represents repeatability. For a more detailed description of other possible coefficients and their relationships, see the study of Feitsma *et al.* (2011). Reliability of CASA is positively related to repeatability, which is increased with a higher number of successfully analysed spermatozoa, thus with decreasing coefficient of variation. Subsequently, in respect of this costly investment, there is a need for economic evaluation together with judging the level of lab technicians' skills in order to achieve the expected trustworthy outputs (Feitsma *et al.* 2011).

Introduction of CASA requires a higher level of economic and technical evaluation for determination of repeatability, calculation of economic profit, as well as training courses for laboratory technicians. Moreover, there is the consideration whether one CASA system would be sufficient for the analysis of a huge number of samples (Feitsma *et al.* 2011). Owing to these facts, a decision to purchase CASA has to be based on technical and economic analyses. Furthermore, managerial staff has to be confident of having skilled employees with the natural undertaking to be further trained, so that the required reliability of results is ensured. Recently, CASA systems have more often been introduced into boar and stallion insemination centres. Nevertheless, there has also been the same tendency in bull insemination centres (Amann and Waberski 2014).

The CASA system was first introduced in 1980 (Mortimer 2000), and over the years it has been developed from the analysis of microphotographs exposition, through analysis of records from each video record, to the latest semi–automatic methods (Rijsselaere *et al.* 2012). The first available systems were the CellSoft Automated Semen Analyser and Hamilton Thorn Motility Analyser (HTM), after which other types of this system were also presented (Katila 2001). The arrangements of CASA systems differ from each other mainly in hardware, optics (microscope, lens, camera) and software accessories (Verstegen *et al.* 2002). The CASA system is based on acquiring consecutive frames from a microscope by means of a simple chip camera (Quintero-Moreno *et al.* 2003). The image is consequently converted to a black/white resolution, exported to the computer and evaluated by specific software with the CASA module, which is able to analyse these images (Mortimer 2000). The obtained data are subsequently mathematically processed and trajectories are defined in numerical form. The results from this operation are reflected as a series of parameters which accurately define the motion of each spermatic cell (Quintero-Moreno *et al.* 2003). The process of motility analysis by CASA is partially automated, but its reliability depends on several factors.

The objective of this review is to summarise and provide complex information about possible errors and difficulties during the analysis of samples and subsequent interpretation of the obtained results. In addition, it reflects a possible relationship between the sperm motility parameters assessed by CASA and their value for estimation or prediction of bull field fertility.

Evaluation of sperm motility by CASA

In general, sperm motility has been considered as one of the most important characteristics in connection with fertilizing ability (Rodriguez-Martinez 2006, Awad 2011). With respect to the fact that cryopreserved semen is used entirely in bovine reproduction, estimation of the motility before cryopreservation closely relates to the quality of spermatozoa post-thaw (Fitzpatrick *et al.* 2002). Subjective motility assessment is considerably affected by the technicians' experience, thus there is a higher probability of the incidence of systematic mistakes (Januskauskas *et al.* 1999, Amann and Waberski 2014). Evaluation of motion characteristics by the CASA system provides a wide scale of kinematic parameters described by Mortimer (2000), which can be seen in Table 1 and are graphically illustrated in Fig. 1. These indicators have a high informative value, characterising the physiological state of spermatozoa and their possible fertilizing ability; thus they provide more accurate information with regard to the estimation of bull fertility (Farrell *et al.* 1998, Kathiravan *et al.* 2011). Because

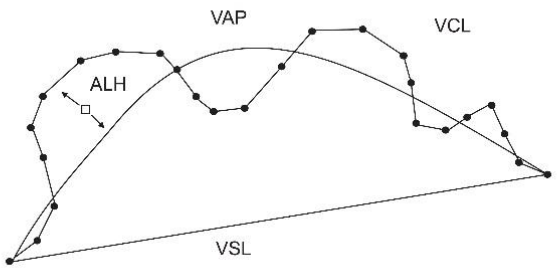


Fig. 1. Expression of selected movement characteristics.

Table 1. Abbreviations used

VCL	curvilinear velocity
VAP	average path velocity
VSL	straight line velocity
STR	straightness (VSL/VAP)
LIN	linearity (VSL/VCL)
WOB	wobble (VAP/VCL)
TMOT	total motility
PMOT	progressive motility
ALH	amplitude of lateral head displacement
BCF	beat cross frequency
LVV	low VAP cut-off
MVV	medium VAP cut-off

there is no standard for animal spermatozoa assessment (Amann and Katz 2004, Feitsma *et al.* 2011), it could be divided according to the World Health Organization (WHO) classification for human spermatozoa into 4 categories – fast, medium fast, slow and immotile. Categorization according to WHO is performed, depending on the lowest and middle value of VAP (LVV and MVV) and the minimal value of STR, in which fast sperms are considered sperms with VAP > MVV, middle fast LVV < VAP < MVV, slow VAP < LVV and immotile without any movement during the analysis. The percentage of sperm with progressive motility (PMOT) is consequently established by VAP which exceeds MVV and the minimal value of STR (WHO 2010). In bull sperm, the accurate values for categorization have not yet been established. This fact is evident in studies by Farrell *et al.* (1998);, Gillan *et al.* (2008), Contri *et al.* (2010), Shojaei *et al.* (2012) and Oliveira *et al.* (2013), where, despite the fact that the authors used the CASA system from the same manufacturer, the values for categorization were set at different levels.

Another method for distinction of the different sperm groups is determination of subpopulations, which are differentiated by cluster analysis. Identification of subpopulations in sperm sample is recently accepted by a wide range of scientists, due to the more meaningful approach to establish a ratio of specific subpopulations in sperm sample in contrast to values of means or medians (Amann and Waberski 2014). In addition to other mammalian species (Abaigar *et al.* 1999, Abaigar *et al.* 2001, Quintero-Moreno *et al.* 2003, Quintero-Moreno *et al.* 2004, Quintero-Moreno *et al.* 2007,), the presence of these subpopulations has also been ascertained in bulls (Muino *et al.* 2008, Muino *et al.* 2009). More importantly, results of studies (Muino *et al.* 2008, Muino *et al.* 2009) showed possible similarities in the distribution of subpopulations among bull sires, thus that subpopulation structure could be constant for bovine sperm samples (Muino *et al.* 2009). The diversity of these subpopulations is a reflection of the differences in morphology, motility, longevity and finally also in their fertilizing ability (Muino *et al.* 2008, Amann and Waberski 2014, Martinez-Pastor *et al.* 2011). Muino *et al.* (2009) reported the presence of the following four sperm subpopulations: the first subpopulation with a relatively low velocity but high progressivity (high LIN, STR, WOB, BCF and low ALH); the second subpopulation which was characterized by high activity but without PMOT, with high values of VCL and ALH simultaneously with low LIN, and STR so-called hyperactivated motility; the third included sperms with very low motility and without progressive movement, which is characterized by the lowest values of VCL, VSL, VAP, ALH, BCF, LIN, STR, WOB; and the fourth subpopulation represented spermatozoa with fast and PMOT with the highest values of both VCL, VSL, VAP, BCF and LIN, STR, WOB, ALH.

From the viewpoint of bovine insemination doses production, it was shown that the percentage of sperm with fast and progressive motility before cryopreservation correlates with the longevity of spermatozoa after thawing (Nunez-Martinez *et al.* 2006). This result indicates that a higher resistance of the ejaculate to the cryopreservation process is linked to a higher incidence of spermatozoa from this subpopulation before freezing. Nunez-Martinez *et al.* (2006), who evaluated Holstein bulls, concluded that the ratio of each subpopulation was similar in both native ejaculate and insemination dose after thawing. Only the case of the percentage of fast sperms with progressive motility was found by these authors as significantly different. Similar results were published in a study where the authors assessed post-thaw motility in a group of Asturiana de los Valles beef cattle (Muino *et al.* 2009).

Factors affecting analysis outputs

Processing of sample: A very important factor for the standardization of the CASA system results is the pre–analytical part, which includes the preparation of a sample for analysis (Amann and Waberski 2014). Thus the concentration, sample volume, counting chamber type, temperature and type of extender are crucial for correct interpretation of CASA results.

The samples have to be diluted before analysis, since the concentration of the native ejaculate is too high for a successful analysis of the pathway of each sperm (Verstegen *et al.* 2002). These authors also indicate that the optimum concentration of sperm for CASA analysis is within the range of 30-50 × 10^6/ml. Additionally, Contri *et al.* (2010) reported that the optimum concentration as 20 × 10^6/ml, when in their experiments the concentration of 50 × 10^6/ml caused a large amount of erroneously generated trajectories and inferior sperm detection. The sample volume of semen after dilution used for CASA analysis varies between 4 – 10 µl, depending on the counting chamber used. For Microcell chambers, 7 µl is used; in Makler chambers it is 4 µl (Januskauskas *et al.* 1999), as in the Leja chambers (Shojaei *et al.* 2012).

In the same way that the use of different types of chambers can affect sperm motility parameters (Lenz *et al.* 2011, Gloria *et al.* 2013, Amann and Waberski 2014), the

duration of the analysis can affect the results of the analysis, both the motion parameters and the total percentage of progressive motile spermatozoa. The duration of the analysis should not exceed 5 min (Contri *et al.* 2010). Due to the small volume which is used for the analysis, the samples have to be maximally homogenized and processed gently. For the safety of the objectivity of the results, higher demands are required on the preparation of samples in comparison with subjective analysis (Feitsma *et al.* 2011, Michos *et al.* 2013).

For analysis of insemination dose, the thawing procedure must be taken into account, since a possible effect on some analysed parameters has been shown (Iguer-ouada and Verstegen 2001, Contri *et al.* 2010). Particularly the temperature of thawing and further incubation (37°C) is crucial (Iguer-ouada and Verstegen 2001).

In addition, the fact that the sample already contains an extender plays a role, because no medium has the same properties as the fluids in the female reproductive tract (Amann and Hammerstedt 2002). Therefore it is necessary to know which type of extender was used before the interpretation of the results. In egg yolk extenders, there are objects with a similar size as the sperm cells yolk granules – which can adversely affect the accuracy of results' objectivity (Wall and Foote 1999, Amirat *et al.* 2005) and the percentage of motile spermatozoa as well (Moussa *et al.* 2002). In contrast, the addition of only effective compound of egg yolk can improve post-thaw motility of spermatozoa (Simonik *et al.* 2013) and can lead to better assessment, due to no presence of yolk granules. The higher viscosity in comparison with non-egg yolk types of media is also related to this influence. This fact has been demonstrated by the reduction of speed parameters (VAP, VSL and VCL) and the percentage of fast-moving sperm cells. Conversely, using HEPES or TALP for sample dilution caused increase of the percentage of motile and rapidly moving spermatozoa. In the case of a saline solution, its usage can avoid the compromise of speed parameters, however, the percentage of progressive and moderately fast moving sperm is also lower (Rijsselaere *et al.* 2003). With regard to the comparison of other diluting media and their relation to the analyzed parameters, there were no significant differences between saline solution, physiological buffer saline and Bioxcell®, with the exception of VCL and ALH, which were significantly higher, and LIN, which was lower when using Bioxcell® compared with saline solutions (Contri *et al.* 2010).

Other factors

When the possible effect of camera properties were assessed, a significant influence of lower imaging speed on total motility (TMOT), progressive motility (PMOT), all the kinematic parameters and categories of fast, middle fast, slow and immotile spermatozoa were found upon a comparison among cameras with 30, 45 and 65 fps (Contri *et al.* 2010). The most common sources of errors in CASA systems are inaccurate object recognition, collisions (Rijsselaere *et al.* 2003, Rijsselaere *et al.* 2012), loss of trajectories due to the high sperm concentration, errors during the reconstruction of the conflicting trajectories and their interpolation. However, there are ongoing innovations of the CASA systems, such as the endeavour to determine new algorithms for a more reliable assessment of static objects and collisions. One of the most basic methods of prevention of incorrect results caused by sperm collisions is, as mentioned previously, appropriate sperm concentration (Verstegen *et al.* 2002). Different types of CASA software represent another 'non-human' factor which plays a role in the reliability of results, especially during comparison among laboratories. Software functions on the same principles, but there are differences in the settings and algorithms for generating trajectories and calculating velocities of each spermatozoon (Amann and Waberski 2014). Moreover, variances among CASA in the capability of distinguishing spermatozoa from debris and egg yolk particles are known (Vincent *et al.* 2012). These factors also present possible influences related to the introduction of CASA in scientific laboratories and insemination centres, as well.

As was previously stated, also the human factor can affect results of analysis in different ways, thus specific requirements have to be fulfilled. The lab technician plays a crucial role in these complex conditions of assessment and can influence almost all of the aforementioned factors, in order to keep obtained results reliable and repeatable (Michos *et al.* 2013, Amann and Waberski 2014). Hence, CASA as a sophisticated system places high demands on skilled operators. Moreover, since there is no defined golden standard for methods of animal spermatozoa motility analysis, so establishment of a standard operating procedure for a specific laboratory is recommended. This should be accompanied by determination of repeatability of assessment, when repeatability higher than 95% is feasible (Feitsma *et al.* 2011). In other words, one of the difficulties in the usage of CASA is the human factor. The purchase and establishment of CASA not only entail maintaining the facilities, but operators need to know the principles of CASA and have to be periodically trained (Michos *et al.* 2013).

Relationship of sperm motility to bull fertility

Motility is accepted as the most important parameter in the set of evaluated characteristics in terms of the fertilization ability of sperm, according to e.g. Fitzpatrick *et al.*(2002) and Kathiravan *et al.* (2011). A number of scientific laboratories deal with the subject of determining one particular parameter, evaluated by the CASA system, and its close correlation with fertility *in vivo*. However, this parameter or laboratory test, which could accurately report information on the fertilization ability of insemination dose, has not yet been established (Oliveira *et al.* 2013). To a certain extent, this cannot be expected due to the fact that the reproductive process is very complex (Feitsma *et al.* 2011, Vincent *et al.* 2012, Amann and Waberski 2014, Ferraz

et al. 2014) and can be affected by different physiological conditions (Rehak *et al.* 2012).

Nevertheless, different sets of specific parameters of motility and their relationship between *in vitro* and *in vivo* fertility were described earlier (Farrell *et al.* 1998, Gillan *et al.* 2008, Oliveira *et al.* 2013). One of the first studies to demonstrate the relationship among kinematic parameters of spermatozoa assessed by CASA and bull fertility was published by Farrell *et al.* (1998). This study was performed on the basis of motility parameters of native ejaculates from 11 bulls and their lifetime fertility. Results indicated that, for the prediction of bull fertility, the following combinations of parameters had the highest correlation values - ALH, BCF, LIN, VAP, VSL ($r^2 = 0.98$); BCF, LIN, VSL, VCL, STR ($r^2 = 0.97$); BCF, LIN, VAP, VSL, STR ($r^2 = 0.97$) and BCF, LIN, VAP, VSL, VCL ($r^2 = 0.97$). Conversely, for single parameters r^2 values were much lower, for example for total motility, the coefficient of determination was 0.34. Similarly, the fact that a combination of several parameters correlated more with *in vivo* fertility was confirmed by Oliveira *et al.* (2013). However, this study was carried out on the basis of evaluation of sperm parameters of frozen-thawed insemination doses and a field artificial insemination programme. Although these authors used the same type of CASA system, the settings were different and, moreover, samples were stained with Hoechst 342 for better visualisation and differentiation of spermatozoa in milk-based extender. In contrast to the study of Farrel *et al.* (1998), partial least squares analysis was applied for the first time for this purpose. Oliveira *et al.* (2013) revealed that motility parameters, which could be used as predictors of field fertility immediately after thawing, were total and progressive motility. After two hours of thermal resistance test, important predictors of fertility were total motility, progressive motility, BCF and percentage of rapidly moving cells. On the basis of these two studies, the fact that more parameters have higher predictive value was confirmed. Moreover, it was found that after 2 hr incubation of frozen-thawed insemination doses, a higher number of evaluated sperm motion characteristics had greater predicative capability.

Zhang *et al.* (1998) also assessed sperm qualitative parameters post-thaw and their possible correlations to bull field fertility. They presented a significant positive correlation of sperm with linear movement pattern. On the other hand, different CASA software was used and, moreover, with a different setting. Another study by Cseh *et al.* (2004), conducted with a different CASA system, showed a correlation with strong significance for VAP and bulls with higher *in vivo* fertility. Cseh *et al.* (2004) proved that lower values of VCL (22.51 – 79.5 μm/s); VSL (11.35 – 35.71 μm/s) and VAP (12.67 – 34.45 μm/s) were linked to bulls with low field fertility.

Contrary to the results of Farrel *et al.* (1998), Cseh *et al.* (2004) and Oliveira *et al.* 2013), the results of Gillan *et al.* (2008) were different. They worked with the same CASA system as Oliveira *et al.* (2013) with almost the same settings, however with a higher number of bulls. Fertility of bulls was assessed with FERTSOL expressing fertility by 56-day non-return rate adjusted for season of insemination, inseminator, and number of inseminations, etc. (Vincent *et al.* 2012). Different groups of bulls were involved to experiment according to these statistically derived fertility values. Gillan *et al.* (2008) showed no correlation of almost all the parameters evaluated by CASA and *in vivo* fertility. Interestingly, results also showed that merely VSL, VCL, ALH, BCF parameters were involved in certain combinations with other specific *in vitro* assessed parameters which had high correlation values > r^2 0.986 which is in contrast to other studies (Farrell *et al.* 1998, Cseh *et al.* 2004, Oliveira *et al.* 2013). But like that of Oliveira *et al.* (2013), this study proved that an increased number of tests resulted in greater correlation values with fertility *in vivo*. Even more relationships among motility parameters and other *in vitro* tests were determined. Oliveira *et al.* (2012) also showed discrepancies in relation to the motility parameters assessed by CASA, when bulls characterized with higher field fertility displayed lower VSL, STR and LIN. On the other hand, bulls with lower fertility had lower TMOT. This fact was additionally supplemented with lower values of parameters TMOT, PMOT, VAP, VSL, VCL, ALH, BCF, STR, LIN after 4 hours of thermo resistance test. Nonetheless, it must be taken account that the study included only three bulls and inter-bull differences were not significant.

Factors which can also play a role in determining a set of indicators for predicting fertility of insemination doses are individual differences in sensitivity of ejaculates to cryopreservation (Muino *et al.* 2008) and interbreed differences (Hoflack *et al.* 2007, Beran *et al.* 2011). Hoflack *et al.* (2007) are some of the few researchers to have investigated a comparison of parameters in native semen of different bull sires – Holstein and Belgian Blue-White (*Bos taurus*). Differences were determined in speed as well as in other physical characteristics. In Belgian Blue-White breed, VCL, VSL and VAP were significantly lower, together with the differences in the kinetic characteristics of ALH and BCF, showing lower movement efficiency. Those differences may also be related to the fertilization ability of sperm after cryopreservation, which was demonstrated by Farrell *et al.* (1998). In the view of the categories, slow, immotile spermatozoa were found in a higher proportion. Moreover, overall and progressively motile sperms were lower as well, in the case of the Belgian Blue-White breed.

Studies to investigate the possible connection of sperm motility assessment before cryopreservation with the quality of insemination doses post-thaw, which is obviously connected with fertilisation ability (Holt 2000), were performed by Muino *et al.* (2008) and Muino *et al.* (2009). Muino *et al.* (2008) presented data of the Holstein breed, which suggested a possible relationship of sperm subpopulations before freezing and their quality post-thaw.

Occurrence of these different sperm subpopulations in the native ejaculate can be used to predict resistance to cryopreservation, in other words, fertilizing ability of the insemination dose. The specific prevalent category of rapidly and progressively moving spermatozoa before cryopreservation is directly proportional to this subpopulation after thawing (Muino *et al.* 2008). In addition, evaluation of the cryopreservation effect on sperm subpopulations of the autochthonous breed of Asturiana de los Valles bulls by Muino *et al.* (2009) proved virtually the same results. The lack of studies concerning differences among other breeds raises the question: what will the situation be when comparing more similar breeds? Are the number and characteristics of sperm subpopulations constant for bovine ejaculates?

Another indicator of bull fertility *in vivo* could be the ability of spermatozoa to transform to the hyperactivation state, thus the ratio of sperm cells with motility pattern which classifies them into so called 'transition phase' (Shojaei *et al.* 2012). This movement pattern was characterized by higher VCL, ALH and simultaneously lower LIN, WOB (Mortimer 2000). The full physiological significance of the hyperactivation phenomenon has not been completely understood. However, there is a presumption that is important for the release of the sperms attached to epithelial cells in the isthmus of the oviduct (Verstegen *et al.* 2002, Marquez and Suarez 2007, Suarez 2008a, b). An essential requirement from the point of view of more precise identification of this sperm movement pattern is to use a camera with more than 60 fps. Cut-offs for recognition of the transition phase were: ALH > 7 μm, LIN < 0.65 and VCL > 80 μm/s (Marquez and Suarez 2007). The ability to use the ratio of spermatozoa in the transition phase determined by CASA to predict field bull fertility was proven by Shojaei *et al.* (2012). These authors showed that the aforementioned values were characteristic for the ejaculate of bulls with higher FERTSOL index, suggesting positive correlation of these motility parameters with increased fertility. As a consequence, the same authors subsequently confirmed in an environment simulating the female genital tract *in vitro*, that the sperms of these bulls with higher fertility easily cross into a state of hyperactivation. This hyperactivated status probably increases the passage of sperm cells through the female reproductive tract to the point of fertilization (Suarez 2008), thus the ability to undergo a hyperactive state could be an indicator of the spermatozoa performance characteristics associated with fertility *in vivo* suggested by Shojaei *et al.* (2012).

Other utilization of CASA

In addition to motility assessment, most CASA systems can be used for an evaluation of sperm concentration, sperm morphology and sperm viability. However, Verstegen *et al.* (2002) reported that, where this parameter was investigated, an accurate assessment of sperm concentration using CASA is a problem in most species. The main problems are underestimation of the number of sperms (Maes *et al.* 2010) and overestimation of the sperm numbers due to collisions and subsequent re-counting of already analyzed objects (Coetzee *et al.* 2001). A major factor which can cause non-uniformity of sperm distribution in a sample, thus the estimation of sperm number, is the counting chamber. After filling, the Segre-Silberberg effect causes excessive sperm movement towards the walls of the chamber, dependent on the Z axis and viscosity of the suspension. It can be reduced by analysing the sample along the central axis of the chamber and also by the use of a correction coefficient (Kuster 2005 , Douglas-Hamilton *et al.* 2005).This physical phenomenon also affects the number of sperms in relation to the time when motile sperms relocate from the walls to the centre of the chamber and immotile sperms cannot be redistributed (Amann and Waberski 2014). In haemocytometer count, which is generally accepted as a gold standard for the determination of sperm concentration, the Segre-Silberberg effect is eliminated due to its depth (100μm). However, the fact that the Bürker chamber has a five times greater depth does not allow its use during CASA concentration evaluation (Hansen *et al.* 2006). These authors presented that the highest agreement for diluted semen was between sperm vision and haemetocytometer. Nonetheless, huge discrepancies can also be found among CASA systems with regard to SpermVision and UltiMate (Douglas and Hamilton 2005). Alternatively, during the comparison of CASA and the subjective evaluation, there has been an overestimation prior to cryopreservation, with the opposite error after thawing. The reason could be the tendency of sperm cells to agglutinate after the cryopreservation process. Another influencing factor may be the system setup for evaluation of native and cryopreserved semen in relation to the used extender (Verstegen *et al.* 2002, Hansen *et al.* 2006). Finally, in bull artificial insemination centres, sperm concentration determination by CASA has been considered as too inaccurate in comparison with standard and routinely used methods. So, the use of the CASA tool is more common in centres where stallion or boar semen is processed (Amann and Waberski 2014). In the case of morphology analysis, Björndahl *et al.* (2010) reported that the current generation of CASA instruments are not able to analyze sperm morphology to a degree that would be suitable for routine use. The problem is the lack of the ability to determine the central part of a sperm flagellum. In subjective evaluation of human sperm morphology, the guidelines are set by WHO, and despite this fact, there can be considerable variability among laboratory technicians and also among all laboratories (Rijsselaere *et al.* 2012, Amann and Waberski 2014). Standardization is not established in morphology evaluation of livestock sperms (Verstegen *et al.* 2002), therefore it is possible to expect even greater variability. Morphology analysis using CASA is also influenced by factors which can distort results, but these can be eliminated by following the standard procedures (Gravance *et al.* 1996, Verstegen *et al.* 2002). Samples for

analysis have to be prepared by washing, staining and fixing before image recording and processing, but this is associated with a greater time consumption and varies according to the CASA system used. Therefore, for maximum image quality, the outputs of quality, type of staining, magnification and the optical device are crucial. These factors, together with different user settings of software may again be a source of variability (Verstegen *et al.* 2002).

Conclusion

In summary, CASA is known and used in laboratories, however certain problems persist. Sperm motility is one of the parameters evaluated routinely. CASA represents a tool for more objective and accurate analysis of sperm motion than subjective evaluation. It must be borne in mind that various human and non-human factors can influence the advisability and objectivity of outputs obtained by CASA. Although CASA provides many parameters for every recognized sperm cell, only a specific approach ensures the meaningful value of a vast number of data for semen evaluation and prediction of bull fertility. Nevertheless, accurate significance of motility parameters to prediction of field fertility has not yet been elucidated.

In all honesty, the use of CASA currently does not represent a tool for precise prediction of bull field fertility. However, a debatable question is: Does it make it possible to discover one or more *in vitro* parameters serving to foretell bull fertility? Outputs of CASA are unlikely going to be individually representative indicators for bull fertility *in vivo*. Nevertheless, specific combinations of motility parameters and defined subpopulations of spermatozoa assessed by CASA should be used together with outputs of other laboratory tests. In this way, they can serve as parameters for improving and increasing the value of *in vitro* assessments for the prediction bull field fertility.

Differences among CASA systems represent obstacles in view of the objective comparison of results across laboratories and also studies investigating prediction values of CASA outputs to bull field fertility. Creation of standards for animal sperm motility assessment by CASA regarding algorithms, parameters, precise object recognition and threshold values for subpopulations can overcome the aforementioned loophole. Moreover, the possible contribution of CASA results to the prediction of bull *in vivo* fertility demands further extensive investigation.

ACKNOWLEDGEMENT

This work was supported by "S" grant of Ministry of Education Youth and Sports and project NAZV - QJ1210109 of the National Agency for Agriculture.

REFERENCES

Abaigar T, Holt W V, Harrison R A P and del Barrio G. 1999. Sperm subpopulations in boar (Sus scrofa) and gazelle (Gazella dama mhorr) semen as revealed by pattern analysis of computer-assisted motility assessments. *Biology of Reproduction* **60** (1): 32–41.

Abaigar T, Cano M, Pickard A R and Holt W V. 2001. Use of computer-assisted sperm motility assessment and multivariate pattern analysis to characterize ejaculate quality in Mohor gazelles (Gazella dama mhorr): effects of body weight, electroejaculation technique and short-term semen storage. *Reproduction* **122** (2): 265–73.

Amann R P and Hammerstedt R H. 2002. Detection of differences in fertility - Andrology lab corner. *Journal of Andrology* **23** (3): 317–25.

Amann R P and Katz D F. 2004. Reflections on CASA after 25 years. *Journal of Andrology* **25** (3): 317–25.

Amann R P and Waberski D. 2014. Computer-assisted sperm analysis (CASA): Capabilities and potential developments. *Theriogenology* **81** (1): 5–17.

Amirat L, Anton M, Tainturier D, Chatagnon G, Battut I and Courtens J L. 2005. Modifications of bull spermatozoa induced by three extenders: Biociphos, low density lipoprotein and Triladyl, before, during and after freezing and thawing. *Reproduction* **129** (4): 535–43.

Awad M M. 2011. Effect of some permaeating cryoprotectants on CASA motility results in cryopreserved bull spermatozoa. *Animal Reproductione Science* **123**: 157–62

Beran J, Stádník L, Ducháèek J, Toušová R, Louda F and Štolc L. 2011. Effect of bulls' breed, age and body condition score on quantitative and qualitative traits of their semen. *Acta universitatis agriculturae et silivicultурae mendelianae brunensis* **54**: 37–44.

Beran J, Stádník L, Bezdíèek J, Louda F, Èítek J andDucháèek J. 2012. Effect of sire and extender on sperm motility and share of live or dead sperm in bulls' fresh ejaculate and in AI doses after thawing. *Archiv Tierzucht* **55**: 207–18.

Beran J, Šimoník O, Stádník L, Bezdíèek J, Louda F and Ducháèek J. 2013. Effect of bull, diluter and LDL – cholesterol concentration on spermatozoa resistance against cold shock. *Acta universitatis agriculturae et silivicultурae mendelianae brunensis* **56:** 1575–81.

Björndahl L, Mortimer D, Barratt Ch L R, Castilla J A, Menkveld R, Alvarez J G, Haugen T B. 2010. A Practical Guide to Basic Laboratory Andrology, 348. Cambridge University Press, Cambridge, United Kingdom.

Coetzee K, Bermes N, Krause W and Menkveld R. 2001. Comparison of normal sperm morphology outcomes from two different computer-assisted semen analysis systems. *Andrologia* **33** (3): 159–63.

Contri A, Valorz C, Faustini M, Wegher L and Carluccio A. 2010. Effect of semen preparation on casa motility results in cryopreserved bull spermatozoa. *Theriogenology* **74** (3): 424–35.

Douglas-Hamilton D H, Smith N G, Kuster C E, Vermeiden J P W and Althouse G C. 2005. Particle distribution in low-volume capillary-loaded chambers. *Journal of Andrology* **26** (1): 107–14.

Farrell P B, Presicce G A, Brockett C C and Foote R H. 1998. Quantification of bull sperm characteristics measured by computer-assisted sperm analysis (CASA) and the relationship to fertility. *Theriogenology* **49** (4): 871–79.

Feitsma H, Broekhuijse M L W J and Gadella B M. 2011. Do CASA Systems Satisfy Consumers Demands? A Critical Analysis. *Reproduction in Domestic Animals* **46** 49–51.

Ferraz M, Morato R, Yeste M, Arcarons N, Pena A I, Tamargo C, Hidalgo C O, Muino R and Mogas T. 2014. Evaluation of sperm subpopulation structure in relation to in vitro sperm-oocyte interaction of frozen-thawed semen from Holstein bulls.

Theriogenology **81** (8): 1067–72.

Fitzpatrick L A, Fordyce G, McGowan M R, Bertram J D, Doogan V J, De Faveri J, Miller R G and Holroyd R G. 2002. Bull selection and use in northern Australia. Part 2. Semen traits. *Animal Reproduction Science* **71** (1-2): 39–49.

Gillan L, Kroetsch T, Maxwell W M C and Evans G. 2008. Assessment of in vitro sperm characteristics in relation to fertility in dairy bulls. *Animal Reproduction Science* **103** (3-4): 201–14.

Gloria A, Carluccio A, Contri A, Wegher L, Valorz C and Robbe D. 2013. The effect of the chamber on kinetic results in cryopreserved bull spermatozoa. *Andrology* **1** (6): 879–85.

Gravance C G, Vishwanath R, Pitt C and Casey P J. 1996. Computer automated morphometric analysis of bull sperm heads. *Theriogenology* **46** (7): 1205–15.

Gravance C G, Casey M E and Casey P J. 2009. Pre-freeze bull sperm head morphometry related to post-thaw fertility. *Animal Reproduction Science* **114** (1-3): 81–88.

Hansen C, Vermeiden T, Vermeiden J P W, Simmet C, Day BC and Feitsma H. 2006. Comparison of FACSCount AF system, improved neubauer hemocytometer, Corning 254 photometer, SpermVision, UltiMate and NucleoCounter SP-100 for determination of sperm concentration of boar semen. *Theriogenology* **66**: 2188–94.

Hoflack G, Opsomer G, Rijsselaere T, Van Soom A, Maes D, de Kruif A and Duchateau L. 2007. Comparison of computer-assisted sperm motility analysis parameters in semen from belgian blue and Holstein-Friesian bulls. *Reproduction in Domestic Animals* **42** (2): 153-61.

Holt W V. 2000. Fundamental aspects of sperm cryobiology: The importance of species and individual differences. *Theriogenology* **53** (1): 47-58.

Iguer-ouada M and Verstegen J P. 2001. Evaluation of the "Hamilton Thorn computer-based automated system" for dog semen analysis. *Theriogenology* **55** (3): 733-49.

Januskauskas A, Gil J, Soderquist L, Haard M G M, Haard M C, Johannisson A and Rodriguez-Martinez H. 1999. Effect of cooling rates on post-thaw sperm motility, membrane integrity, capacitation status and fertility of dairy bull semen used for artificial insemination in Sweden. *Theriogenology* **52** (4): 641-58.

Kathiravan P, Kalatharan J, Edwin M J and Veerapandian C. 2008. Computer automated motion analysis of crossbred bull spermatozoa and its relationship with in vitro fertility in zona-free hamster oocytes. *Animal Reproduction Science* **104** (1): 9-17.

Kathiravan P, Kalatharan J, Karthikeya G, Rengarajan K and Kadirvel G. 2011. Objective Sperm Motion Analysis to Assess Dairy Bull Fertility Using Computer-Aided System - A Review. *Reproduction in Domestic Animals* **46** (1): 165-72.

Katila T. 2001. In vitro evaluation of frozen-thawed stallion semen: A review. *Acta Veterinaria Scandinavica* **42** (2): 199.

Kuster C. 2005. Sperm concentration determination between hemacytometric and CASA systems: Why they can be different. Theriogenology 64 (3): 614-17.

Lenz R W, Kjelland M E, VonderHaar K, Swannack T M and Moreno J F. 2011. A comparison of bovine seminal quality assessments using different viewing chambers with a computer-assisted semen analyzer. *Journal of Animal Science* **89** (2): 383-88.

Lu J C, Huang Y F and Lu N Q. 2014. Computer-aided sperm analysis: past, present and future. *Andrologia* **46** (4): 329-38.

Maes D, Rijsselaere T, Vyt P, Sokolowska A, Deley W and Van Soom A. 2010. Comparison of five different methods to assess the concentration of boar semen. *Vlaams Diergeneeskundig Tijdschrift* **79** (1): 42-47.

Marquez B and Suarez S S. 2007. Bovine sperm hyperactivation is promoted by alkaline-stimulated Ca2+ influx. *Biology of Reproduction* **76** (4): 660-65.

Martinez-Pastor F, Jorge Tizado E, Julian Garde J, Anel L and de Paz P. 2011. Statistical Series: Opportunities and challenges of sperm motility subpopulation analysis. *Theriogenology* **75** (5): 783-95.

Michos I A, Basioura A G, Boscos C M and Tsakmakidis I A. 2013. Proper use and impact of 'Computer Assisted Semen Analysis' technique on semen evaluation of farm animals. *Journal of the Hellenic Veterinary Medical Society* **64** (4): 267-74.

Mortimer S T. 1997. A critical review of the physiological importance and analysis of sperm movement in mammals. *Human Reproduction Update* **3** (5): 403-39.

Mortimer S T. 2000. CASA - Practical aspects. *Journal of Andrology* **21** (4): 515-24.

Moussa M, Martinet V, Trimeche A, Tainturier D and Anton M. 2002. Low density lipoproteins extracted from hen egg yolk by an easy method: cryoprotective effect on frozen-thawed bull semen. *Theriogenology* **57** (6): 1695-706.

Muino R, Tamargo C, Hidalgo C O and Pena A I. 2008. Identification of sperm subpopulations with defined motility characteristics in ejaculates from Holstein bulls: Effects of cryopreservation and between-bull variation. *Animal Reproduction Science* **109** (1-4): 27-39.

Muino R, Pena A I, Rodriguez A, Tamargo C and Hidalgo C O. 2009. Effects of cryopreservation on the motile sperm subpopulations in semen from Asturiana de los Valles bulls. *Theriogenology* **72** (6): 860-68.

Nunez-Martinez I, Moran J M and Pena F J. 2006. A three-step statistical procedure to identify sperm kinematic subpopulations in canine ejaculates: Changes after cryopreservation. *Reproduction in Domestic Animals* **41** (5): 408-15.

Oliveira L Z, de Arruda R P, de Andrade A F C, Celeghini E C C, dos Santos R M, Beletti M E, Peres R F G, Oliveira C S and de Lima V. 2012. Assessment of field fertility and several in vitro sperm characteristics following the use of different Angus sires in a timed-AI program with suckled Nelore cows. *Livestock Science* **146** (1): 38-46.

Oliveira L Z, de Arruda R P, de Andrade A F C, Celeghini E C C, Reeb P D, Martins J P N, dos Santos R M, Beletti M E, Peres R F G, Monteiro F M and de Lima V. 2013. Assessment of in vitro sperm characteristics and their importance in the prediction of conception rate in a bovine timed-AI program. *Animal Reproduction Science* **137** (3-4): 145-55.

Puglisi R, Pozzi A, Foglio L, Spano M, Eleuteri P, Grollino M G, Bongioni G and Galli A. 2012. The usefulness of combining traditional sperm assessments with in vitro heterospermic insemination to identify bulls of low fertility as estimated in vivo. *Animal Reproduction Science* **132** (1-2): 17-28.

Quintero-Moreno A, Miro J, Rigau A T and Rodriguez-Gil J E. 2003. Identification of sperm subpopulations with specific motility characteristics in stallion ejaculates. *Theriogenology* **59** (9): 1973-90.

Quintero-Moreno A, Rigau T and Rodriguez-Gil J E. 2004. Regression analyses and motile sperm subpopulation structure study as improving tools in boar semen quality analysis. *Theriogenology* **61** (4): 673-90.

Quintero-Moreno A, Rigau T and Rodriguez-Gil J E. 2007. Multivariate cluster analysis regression procedures as tools to identify motile sperm subpopulations in rabbit semen and to predict semen fertility and litter size. *Reproduction in Domestic Animals* **42** (3): 312-19.

Rehak D, Volek J, Barton L, Vodkova Z, Kubesova M and Rajmon R. 2012. Relationships among milk yield, body weight, and reproduction in Holstein and Czech Fleckvieh cows. *Czech Journal of Animal Science* **57** (6): 274-82.

Rijsselaere T, Van Soom A, Maes D and de Kruif A. 2003. Effect of technical settings on canine semen motility parameters measured by the Hamilton-Thorne analyzer. *Theriogenology* **60** (8): 1553-68.

Rijsselaere T, Van Soom A, Maes D and Nizanski W. 2012. Computer-Assisted Sperm Analysis in Dogs and Cats: An Update after 20 Years. *Reproduction in Domestic Animals* **47** 204-07.

Rodriguez-Martinez H. 2006. Can we increase the estimative value of semen assessment? *Reproduction in Domestic Animals* **41** 2-10.

Shojaei H, Kroetsch T, Wilde R, Blondin P, Kastelic J P and Thundathil J C. 2012. Moribund sperm in frozen-thawed semen, and sperm motion end points post-thaw and post-swim-up, are related to fertility in Holstein AI bulls. *Theriogenology* **77** (5): 940-51.

Suarez S S. 2008a. Control of hyperactivation in sperm. *Human Reproduction Update* **14** (6): 647-57.

Suarez S S. 2008b. Regulation of sperm storage and movement in the mammalian oviduct. *International Journal of Developmental Biology* **52** (5-6): 455–62.

Sundararaman M N, Kalatharan J and Thilak Pon Jawahar K. 2012. Computer asissted semen analysis for quantification of motion characteristics of bull sperm during cryopreservation cycle. *Veterinary World* **5**: 723–726.

Simonik O, Stadnik L, Rajmon R, Beran J, Sichtar J and Duchacek J. 2013. Influence of LDL addition on cryoprotective properties of bovine semen extenders. Proceedings of 4th International Agronomic Symposium "Agrosym 2013". 3–6 October 2013. Jahorina, Bosnia and Herzegovina.

Verstegen J, Iguer-Ouada M and Onclin K. 2002. Computer assisted semen analyzers in andrology research and veterinary practice. *Theriogenology* **57** (1): 149–79.

Vincent P, Underwood S L, Dolbec C, Bouchard N, Kroetsch T and Blondin P. 2012. Bovine semen quality control in artificial insemination centres. *Animal Reproduction* **9**: 153–165.

Wall R J and Foote R H. 1999. Fertility of bull sperm frozen and stored in clarified egg yolk-Tris-glycerol extender. *Journal of Dairy Science* **82** (4): 817–21.

WHO. 2010. WHO Laboratory Manual for the Examination and processing of human semen. 5th edition, p 138. Cambridge University Press, Cambridge, UK.

Zhang B R, Larsson B, Lundeheim N and Rodriguez-Martinez H. 1998. Sperm characteristics and zona pellucida binding in relation to field fertility of frozen-thawed semen from dairy AI bulls. *International Journal of Andrology* **21** (4): 207–16.

4.2 Ovlivnění rezistence spermií býků vůči chladovému šoku - vliv přítomnosti LDL v ředidlech semene před kryokonzervací

Již v průběhu procesu postupného zchlazování a ekvilibrace, jakožto jednoho z kroků kryokonzervace spermií, dochází ke značné negativní zátěži (Barbas et Mascarenhas, 2009). Jedním z hlavních projevů stresových podmínek jsou změny v lipidové struktuře plazmatické membrány vedoucí k nevratným změnám především, co se týče obsahu cholesterolu (Salmon et al., 2016). Je známo, že tato molekula dokáže s plazmatickou membránou interagovat, je tedy evidentní, že hraje klíčovou roli ve stabilitě plazmatické membrány a ochraně jejich funkčních vlastností (Moce et al., 2010a; Le Guillou et al., 2016).

Hypotéza: Modifikace receptury vybraných ředidel pomocí LDL bude mít pozitivní dopad na rezistenci spermií vůči chladovému šoku.

Cíl: Stanovit efekt LDL v ředidlech semene na rezistenci spermií býků vůči chladovému šoku.

Vliv LDL před vlastní kryokonzervací spermií byl hodnocen na základě jejich rezistence vůči chladovému šoku v průběhu dvouhodinného termodynamického testu supravitálním barvením pomocí eosinu a nigrosinu.

Prvotní výsledky ukázaly srovnatelné procento spermií s intaktní plazmatickou membránou v přítomnosti LDL v porovnání s kontrolními skupinami ředidel na začátku i na konci dvouhodinového termodynamického testu. Na základě podrobnější statistické analýzy, která zohledňovala interakci LDL se specifickým typem ředidla, byl odhalen pozitivní vliv adice 6% LDL do ředidla Bioxcell. Obdobný vliv byl pozorován i po dvou hodinách termodynamického testu, kdy stejná koncentrace LDL největší měrou zredukovala procento spermií s porušenou plazmatickou membránou.

Bylo tedy prokázáno, že již před kryokonzervací LDL vykazuje pozitivní efekt v kontextu použitého ředidla s obsahem sójového lecitinu na plazmatickou membránu spermií. Potvrdil se tak potenciál testování vlivu adice LDL ve smyslu zvyšování kryoprotektivních vlastností této kategorie ředidel.

Publikace:

Beran, J., **Šimoník, O.**, Stádník, L., Rajmon, R., Ducháček, J., Krejcárková, A., Doležalová, M., Šichtař, J. 2013. Effect of bull, diluter and LDL-cholesterol concentration on spermatozoa resistence against cold shock. Acta Universitatis Agriculturae et Silviculturae Mendelianae Brunensis. 61 (6). 1575-1581.

Beran, J., **Šimoník, O.**, Rajmon, R., Stádník, L., Doležalová, M., Krejcárková, A., Ducháček, J., Šichtař, J. 2016. Effect of LDL addition into selected bull sperm diluters on resistance of spermatozoa against cold shock. Acta universitatis agriculturae et silviculturae mendelianae brunensis. 64 (2). 395-399.

ACTA UNIVERSITATIS AGRICULTURAE ET SILVICULTURAE MENDELIANAE BRUNENSIS

Volume LXI 173 Number 6, 2013
http://dx.doi.org/10.11118/actaun201361061575

EFFECT OF BULL, DILUTER AND LDL-CHOLESTEROL CONCENTRATION ON SPERMATOZOA RESISTANCE AGAINST COLD SHOCK

Jan Beran, Ondřej Šimoník, Luděk Stádník, Radko Rajmon, Jaromír Ducháček, Adéla Krejcárková, Martina Doležalová, Jiří Šichtař

Received: August 30, 2013

Abstract

BERAN JAN, ŠIMONÍK ONDŘEJ, STÁDNÍK LUDĚK, RAJMON RADKO, DUCHÁČEK JAROMÍR, KREJCÁRKOVÁ ADÉLA, DOLEŽALOVÁ MARTINA, ŠICHTAŘ JIŘÍ: *Effect of bull, diluter and LDL-cholesterol concentration on spermatozoa resistance against cold shock*. Acta Universitatis Agriculturae et Silviculturae Mendelianae Brunensis, 2013, LXI, No. 6, pp. 1575–1581

The objectives of this study were to determine and evaluate the effect of bull, diluter and addition of LDL in different concentration on the percentage rate of spermatozoa survival after cold shock. In total, four bulls were collected during a period of eight weeks. A total of 8 samples of fresh semen with required quality were processed. Three extenders were used for dilution of each sample; AndroMed®, Bioxcell® and Triladyl®, each in standard and LDL enriched variants. In the case of AndroMed® and Bioxcell®, 4, 6 and 8% of LDL were simply added. In Triladyl®, 6, 8 and 10% of LDL replaced the standard egg yolk component. Resistance of spermatozoa against cold shock (0 °C, 10 minutes) was evaluated by the percentage rate of live sperm using Eosin-Nigrosine staining immediately and 2 hours after heat incubation (37 °C). The results showed the influence of bull individuality as an important factor. Among diluters used it is possible to recommend AndroMed® and Bioxcell® due to significantly ($P < 0.01$) lower decline of live sperm proportion during the cold shock test than Triladyl® (–9.19, respectively –4.95%). The optimal LDL concentration increasing resistance of spermatozoa against cold shock was not determined, therefore subsequent research is necessary.

bull semen, sperm survival, cold shock, extender, LDL cholesterol

Fertility of dairy cows has declined over the past five decades as milk production per cow has increased (Zink *et al.*, 2012; Walsh *et al.*, 2011). The issue of cattle fertility was intensively studied in recent years from cow point of view and effects on their reproductive performance (Doležalová *et al.*, 2013; Roche *et al.*, 2011; Hanuš *et al.*, 2010; LeBlanc *et al.*, 2010). However, cow's reproduction results are also affecting by male component which is represented by the fertilization ability of bull ejaculate.

Sperm quality is influenced by many factors, e.g. by such internal factors as breed, variation between individuals, and age of sire (Beran *et al.*, 2011; Štolc *et al.*, 2009), and by such external factors as environmental conditions (Balic *et al.*, 2012), composition of the diet (Horký *et al.*, 2012) and frequency of collecting ejaculate (Kaya *et al.*, 2002). Collection of ejaculate and its subsequent processing present further potential risk factors for declined sperm quality. Phases of producing insemination doses (diluting of sperm, filling of straws, cooling and freezing) have a significant effect on sperm motility after thawing (Siddique *et al.*, 2006), and the properties of extender used are especially important (Beran *et al.*, 2012; Hegedüšová *et al.*, 2012), as well as method of thawing (Filipčík and Hanuláková, 2011).

Low Density Lipoprotein (LDL cholesterol – LDL) – a component of egg yolk – is believed to

be largely responsible for the egg yolk protective effects on spermatozoa during the freezing process (Pace and Graham, 1974) increasing their resistance against cold shock (Moussa *et al.*, 2002). There is an assumption that extenders containing LDL, which is extracted from egg yolk, can have better effects on spermatozoa membranes during the freezing than diluters commercially exploited (Moussa *et al.*, 2002).

Biological tests of ejaculate have been developed to assess the resistance and the fertilization ability of sperm using LDL addition into diluters, e.g. short-term heat test of sperm survival (Maurya and Tuli, 2003), hypo-osmotic swelling tests (Padrik *et al.*, 2012), however resistance of sperm against cold shock has not been evaluated as well in relation to addition of LDL.

Thus, the aim of this paper was to determine the effect of bull, diluter and addition of LDL in different concentration on the percentage rate of spermatozoa survival after cold shock.

MATERIAL AND METHODS

Semen collecting, dilution and processing

Ejaculate of four bulls (No. 1, 2, 3, and 4) belonging to one artificial insemination (AI) centre approved for public use, were sampled and evaluated. Samples of ejaculate were obtained using an artificial vagina during the period of eight weeks and tested immediately after collection. Volume of semen (VOL), density of sperm (DEN, and percentage of progressive motile spermatozoa above head (ACT) were evaluated by only one trained technician of AI centre. A total of 8 samples of fresh semen with required quality (minimum progressive motility 70% and sperm concentration 0.7 × 10^6 mm^{-3}) were then transferred at 4 °C to the university laboratory within one hour for next processing.

Three extenders were used for dilution of each sample; AndroMed®, Triladyl® (both from MiniTüb GmbH, Tiefenbach, Germany) and Bioxcell® (IMV, L' Aigle, France). Control variants of diluters (0% LDL addition) were prepared according to the manufacturer's instructions. Experimental variants included 4, 6 and 8% LDL addition into the AndroMed® and Bioxcell® diluters, and 6, 8 and 10% LDL addition into the Triladyl® diluter prepared without using egg yolk, which is normally its essential component. Higher concentrations of LDL in Triladyl® replaced cryoprotective properties of egg yolk. The extenders were prepared on the day of sampling and stored at the cooling box (4 °C) before ejaculate dilution.

Samples of semen were pipetted using a sterile pipette to a sterile chilled (4 °C) tubes. Each sample of semen was immediately diluted to 50 000 spermatozoa/ml. The required amount of diluters was applied using sterile syringes directly to the tubes with samples. Thereafter tubes were sealed with sterile stoppers, mixed gently and placed into the cooling box (4 °C).

Evaluation of resistance against cold shock

Three capillaries (0.1 ml) were successively filled from each sample at 4 °C, closed at one end with plasticine and stored at 0 °C into a cooled bath (No Ice, Bibby Scientific, Ltd., Staffordshire, UK) for ten minutes. After the end of cold incubation the capillaries content was gently mixed on preheated hour glass (37 °C) with 20 µl of Eosin by circular motion for 30 sec. and then Nigrosine was added at amount of 40 µl. A volume of 20 µl of the resulting suspension was added into a preheated glass slide and smear was done at the beginning of the test (time 0). This procedure was repeated after 2 hours heat incubation of extended semen samples in a water bath at 37 °C. After drying each smear (72 of each collecting day, 576 together) was examined under a phase contrast microscope (Eclipse E200, Nikon®, Tokyo, Japan) at 1 000x magnification and with oil immersion by only one evaluator. Minimum of 100 spermatozoa was classified as either dead (with red heads) or live (with white heads) and expressed as a percentage rate of live sperm at the beginning of the test (L0) and after 2 hours of heat incubation (L2).

Statistical analysis

The data were evaluated with statistical software SAS 9.3. (SAS/STAT® 9.3, 2011) using UNIVARIATE, CORR, and MIXED procedures. The following equation was used:

$$Y_{ijkl} = \mu + BULL_i + DIL_j + LDLC_k + b_1*(\text{VOL}) + b_2*(\text{DEN}) + b_3*(\text{ACT}) + e_{ijkl},$$

where:

Y_{ijkl} ..... observed value of the dependent variable (percentage rate of live sperm at the beginning of the test and after 2 hours heat incubation, difference between these two measurements),

$BULL_i$ ..... fixed effect of the i^{th} bull (i = 1, n = 216; 2, n = 216; 3, n = 72; 4, n = 72);

DIL_j ..... fixed effect of the j^{th} diluter (j = 1 – AndroMed®, n = 192; 2 – Bioxcell®, n = 192; 3 – Triladyl®, n = 192);

$LDLC_k$ ..... fixed effect of the k^{th} concentration of LDL (k = 0, n = 144; 4, n = 96; 6, n = 144; 8, n = 144; 10, n = 48);

$b_1*(\text{VOL})$ .... regression on volume of ejaculate;

$b_2*(\text{DEN})$ .... regression on density of sperm;

$b_3*(\text{ACT})$ .... regression on activity of sperm;

e_{ijkl} ..... residual effects.

The differences between the variables estimated were tested at the levels of significance $P < 0.05$ and $P < 0.01$. Pearson correlation coefficients were also determined.

RESULTS AND DISCUSSION

The basic statistical characteristics of observed data are shown in Tab. I. The volume of ejaculate

I: *Basic statistical characteristics of observed data*

Variable	Unit	Min	Max	Mean	SD
VOL	[g]	6.2	11.8	9.78	1.663
DEN	[10^6. mm^{-3}]	0.9	1.8	1.28	0.269
ACT	[%]	80	90	87.5	3.542
L0	[%]	23.27	93.2	74.12	16.245
L2	[%]	5.98	89.25	54.37	16.667
L0 – L2	[%]	0.61	54.62	19.68	11.528

Key: VOL = volume of ejaculate; DEN = density of sperm; ACT = activity of sperm; L0 = percentage rate of live sperm at the beginning of the test; L2 = percentage rate of live sperm after 2 hours of the test duration; L0 – L2 = difference between percentage rate of live sperm at time 0 and 2 hours of the test

II: *Pearson correlation coefficients r and related statistical significance P among evaluated traits*

		DEN [10^6. mm^{-3}]	ACT [%]	L0 [%]	L2 [g]	L0 – L2 [%]
VOL [g]	r	0.5886	0.7563	0.8524	0.6006	0.3445
	P	< .0001	< .0001	< .0001	< .0001	< .0001
DEN [10^6. mm^{-3}]	r		0.4616	0.5607	0.3876	0.2377
	P		< .0001	< .0001	<.0001	< .0001
ACT [%]	r			0.8063	0.6007	0.2914
	P			< .0001	< .0001	< .0001
L0 [g]	r				0.7565	0.3256
	P				< .0001	< .0001

Key: VOL = volume of ejaculate; DEN = density of sperm; ACT = activity of sperm; L0 = percentage rate of live sperm at the beginning of the test; L2 = percentage rate of live sperm after 2 hours of the test duration; L0 – L2 = difference between percentage rate of live sperm at time 0 and 2 hours of the test

ranged from 6.2 to 11.8 g in selected bulls. The volumes of ejaculates correspond to the findings of other authors, e.g. Louda *et al.* (2007) reported the range 3–12 g in sires kept in AI centre. Ball and Peters (2004) mentioned the closer range from 5 to 6 g.

The sperm density ranged from 0.9 to 1.8 × 10^6 mm^{-3}. Our results again agreed with those of Louda *et al.* (2007), who reported standard sperm density of bulls' ejaculates from 0.8 to 2.0 × 10^6 mm^{-3}. Ball and Peters (2004) observed a range from 0 in azoospermic bulls to 3 000 × 10^6 mm^{-3} in excellent sires, however especially mentioned substandard marginal bulls could not be used as widely applied sires.

The activity of sperm immediately after collection ranged from 80 to 90% due to determined threshold of 70% commonly used in Czech AI centers. These values correspond with those of Louda *et al.* (2007), who reported sperm activity 45–75% or more. Similarly, Ball and Peters (2004) reported that at least 60% of the spermatozoa should be shown straight progressive movement above head. This requirement was fulfilled within our observation.

Cold shock test belongs to the main evaluation method of sperm quality. We take a reflection about viability and fertility of ejaculate. The rate of live sperm ranged from 23.27 to 93.2% in the beginning and from 5.98 to 89.25% after 2 hours of the heat incubation.

Indicators mentioned above belong to the main characteristics of collected fresh semen (Hanuláková *et al.*, 2012) and determine the initial quality of ejaculate subsequently used for AI doses manufacturing (Vágenknechtová *et al.*, 2011). The last findings confirm that the initial quality of ejaculate determines final quality of AI dose (Beran *et al.*, 2012).

Tab. II contains Pearson correlation coefficients among the evaluated traits. Significant ($P < 0.01$) correlation coefficients ($r = 0.2377$ to 0.8524) were detected between all characteristics evaluated.

Further, the effects of bull, diluter and LDL addition have been evaluated in detail by the statistical model designed. Results of this evaluation are presented in Tab. III and IV. Coefficient of the whole model repeatability ranged from $r^2 = 0.408$ to $r^2 = 0.852$ during the evaluation of observed traits. Effect of bull was significant ($P < 0.01$) in relation to the whole evaluated traits. Effect of diluter was significant ($P < 0.01$) in relation to the percentage rate of live sperm after 2 hours of the heat incubation and difference between percentage rate of live sperm at the beginning and 2 hours of the heat incubation. Effect of LDL concentration in tested samples was insignificant to all evaluated traits ($P > 0.05$). The statistical model also included the effects of the initial quality parameters of ejaculate regression (volume, density and activity). Effect of volume of ejaculate was significant ($P < 0.01$) only in relation

III: *Effects of individual factors in statistical model*

TRAIT	MODEL		BULL		DIL		LDLC		VOL		DEN		ACT	
	r^2	P	F-test	P	F-test	P	F-test	P	F-test	P	F-test	P	F-test	P
L0	0.852	< 0.001	34.03	< 0.001	2.06	0.13	1.20	0.311	9.13	0.003	9.02	0.003	13.38	< 0.001
L2	0.697	< 0.001	62.70	< 0.001	26.61	< 0.001	0.13	0.969	1.20	0.274	9.44	0.002	3.11	0.08
L0 – L2	0.408	< 0.001	25.31	< 0.001	19.38	< 0.001	1.23	0.298	0.68	0.410	27.8	< 0.001	18.85	<0.001

Key: L0 = percentage rate of live sperm at the beginning of the test; L2 = percentage rate of live sperm after 2 hours of the test duration; L0 – L2 = difference between percentage rate of live sperm at time 0 and 2 hours of the test; BULL = effect of each bull; DIL = effect of each diluter; LDLC = effect of different concentration of LDL in tested sample; VOL = volume of ejaculate; DEN = density of sperm; ACT = activity of sperm

IV: *Effect of bull, diluter and concentration of LDL on sperm survivability after cold shock*

EFFECT	LEVEL	L0	L2	L0 – L2
		LSM ± SE	LSM ± SE	LSM ± SE
BULL	1	75.40 ± 1.193^{A}	64.05 ± 1.807^{A}	11.56 ± 1.747^{A}
	2	78.82 ± 1.083^{A}	56.00 ± 1.624^{B}	$22.86 \pm 1.571^{A,B}$
	3	75.70 ± 1.625^{A}	$61.84 \pm 2.408^{A,B}$	$13.67 \pm 2.328^{b,A,B}$
	4	54.66 ± 3.447^{B}	14.60 ± 5.167^{C}	$39.54 \pm 4.996^{b,B,C}$
DIL	AndroMed®	71.65 ± 1.053	54.25 ± 1.556^{A}	17.43 ± 1.504^{A}
	Bioxcell®	71.88 ± 1.053	50.02 ± 1.554^{B}	21.67 ± 1.502^{B}
	Triladyl®	69.91 ± 1.032	43.10 ± 1.531^{C}	26.62 ± 1.481^{C}
LDLC	0	69.87 ± 1.075	49.61 ± 1.597	20.12 ± 1.545
	4	71.27 ± 1.238	49.46 ± 1.840	21.51 ± 1.779
	6	71.26 ± 1.075	48.58 ± 1.587	22.76 ± 1.535
	8	72.18 ± 1.075	48.84 ± 1.597	23.20 ± 1.545
	10	71.16 ± 1.632	49.14 ± 2.404	21.95 ± 2.325

Key: BULL = effect of each bull; DIL = effect of each diluter; LDLC = effect of different concentration of LDL in tested sample; L0 = percentage rate of live sperm at the beginning of the test; L2 = percentage rate of live sperm after 2 hours of the test duration; L0 – L2 = difference between percentage rate of live sperm at time 0 and 2 hours of the test. Different superscript letters mean a significant difference within a column – a, b = $P < 0.05$; A, B, C = $P < 0.01$

to the percentage rate of live sperm at the beginning of the test. This is in accordance with physiological basis, due to amount of sperm and their supply with important substances from seminal plasma. Effect of sperm density was significant ($P < 0.01$) in relation to the whole evaluated traits. This result has also biological background, because density of sperm in ejaculate defines the average conditions for the cells functioning. The effect of sperm activity was significant ($P < 0.01$) to the percentage rate of live sperm at the beginning and difference between percentage rate of live sperm at time 0 and after 2 hours of the heat incubation. This is also logical as sperm activity represents an indicator of initial quality – viability of spermatozoa.

Significant differences ($P < 0.05 – 0.01$) were detected between bulls regardless of the used diluter or LDL concentration. At the beginning of the test was the best bull No. 2 (78.82 ± 1.083%), while after 2 hours had the best survivability bull No. 1 (64.05 ± 1.807%), including the smallest difference between the first and second measurement (11.56 ± 1.747%). Significantly ($P < 0.01$) the lowest values of sperm survivability during whole test had bull number 4 (54.66 ± 3.447, respectively 14.60 ± 5.167%). This bull also had significantly ($P < 0.05–0.01$) the highest sperm survivability decline between time 0 and 2 hours of the heat incubation (39.54 ± 4.996%). Our results confirmed that effect of bull is important, individual differences were found between them (Thara and Nair, 2007).

Focusing on the influence of diluter regardless of the concentration of LDL we can assume that AndroMed® (71.65 ± 1.053%) and Bioxcell® (71.88 ± 1.053%) had the best results at the beginning of the test and Triladyl® (69.91 ± 1.032%) reached the worst results. Triladyl® extender achieved significantly ($P < 0.01$) the worst results after 2 hours of the heat incubation (–6.92% less than Bioxcell® and –11.12% less than AndroMed®). This extender also had significantly ($P < 0.01$) the highest declined percentage rate of live sperm during the entire the test (26.62 ± 1.481%). Our results showed that better results of the cold shock test were achieved using the AndroMed® and Bioxcell®. This is in accordance with Jannet *et al.* (2005) or Stradaioli *et al.* (2007) who state that AndroMed®, respectively Bioxcell® are the most suitable extenders for cryopreservation of bull semen.

In Tab. IV are also shown results of LDL concentration influence on sperm survivability without specification of extender type. Differences between each concentration of LDL cholesterol were statistically insignificant ($P > 0.05$). The lowest value of percentage rate of live sperm (69.87 ± 1.075%) at the beginning of the test had control samples (0% LDL cholesterol). While these samples had the best results at the end of the heat incubation (49.61 ± 1.597%). These samples also had the smallest difference between time 0 and 2 hours of the test. There is need to say that all differences of sperm survivability detected in relation to concentration of LDL were insignificant ($P > 0.05$). The values of percentage rate of live sperm after 2 hours of the heat incubation were almost the same in all LDL concentrations. From these results we cannot clearly determine the most suitable concentration of LDL addition and subsequent research in this area is necessary.

CONCLUSION

Based on our monitoring we can assume that effect of bull is important and individual differences between selected sires in their sperm resistance against cold shock were detected. AndroMed® and Bioxcell® have been found as the more suitable extenders for cryopreservation of bull semen compared to Triladyl® due to lower decline of live sperm proportion during the cold shock test. Effect of LDL concentration added to extenders was insignificant ($P > 0.05$) and showed unclear results. We cannot recommend the optimal LDL concentration increasing resistance of spermatozoa against cold shock. Therefore, subsequent research of this topis is necessary.

SUMMARY

The aim of this study was to determine and evaluate the effect of bull, diluter and addition of LDL in different concentration on the percentage rate of spermatozoa survival after cold shock.

In total, four bulls were collected during a period of eight weeks. A total of 8 samples of fresh semen with required quality were processed. Three extenders were used for dilution of each sample: AndroMed®, Triladyl® (both from MiniTüb GmbH, Tiefenbach, Germany) and Bioxcell® (IMV, L' Aigle, France). In the case of AndroMed® and Bioxcell®, 4, 6 and 8% of LDL were simply added. In Triladyl®, 6, 8 and 10% of LDL replaced the standard egg yolk component. Resistance of spermatozoa against cold shock (0 °C, 10 minutes) was evaluated by the percentage rate of live sperm using Eosin-Nigrosine staining in time 0 and 2 hours of heat incubation (37 °C) after exposure to cold. In total, 576 smears were evaluated. Statistical software SAS 9.3., procedures UNIVARIATE, CORR, and MIXED were used for analyzing the data.

Effect of bull was significant ($P < 0.01$) in relation to the whole evaluated traits. Effect of diluter was significant ($P < 0.01$) in relation to the percentage rate of live sperm after 2 hours of the heat incubation and difference between percentage rate of live sperm at the beginning and 2 hours of the heat incubation. We can recommend AndroMed® and Bioxcell® due to significantly ($P < 0.01$) lower decline of live sperm proportion during the cold shock test than Triladyl® (–9.19, respectively –4,95%). Effect of LDL concentration in tested samples was insignificant to all evaluated traits ($P > 0.05$). We cannot recommend the optimal LDL concentration increasing resistance of spermatozoa against cold shock and subsequent research is necessary.

Acknowledgment

The work was funded by „S" grant of MŠMT ČR and project NAZV QJ1210109. We thank the company Natural Ltd. for cooperation on this work.

REFERENCES

BALIC, I. M., MILINKOVIC-TUR, S., SAMARDZIJA, M., VINCE, S., 2012: Effect of age and environmental factors on semen quality, glutathione peroxidase activity and oxidative parameters in simmental bulls. *Theriogenology*, 78, 2: 423–431. ISSN 0093-691X.

BALL, P. J. H., PETERS, A. R., 2004: *Reproduction in cattle*. 3. vyd. Oxford: Blackwell Publishing, 242 s. ISBN 1-4051-1545-9.

BERAN, J., STÁDNÍK, L., DUCHÁČEK, J., TOUŠOVÁ, R., LOUDA, F., 2011: Effect of bulls' breed, age and body condition score on quantitative and qualitative traits of their semen. *Acta Univ. Agric. et Silvic. Mendel. Brun.*, 59, 6: 37–44. ISSN 1211-8516.

BERAN, J, STÁDNÍK, L., BEZDÍČEK, J., LOUDA, F., ČÍTEK, J., DUCHÁČEK, J., 2012: Effect of sire and extender on sperm motility and share of live or dead sperm in bulls' fresh ejaculate and in AI doses after thawing. *Archiv fur Tierzucht – Archives Animal Breeding*, 55, 3: 207–218. ISSN 0003-9438.

DOLEŽALOVÁ, M., STÁDNÍK, L., NEJDLOVÁ, M., NĚMEČKOVÁ, D., BERAN, J., DUCHÁČEK, J., 2013: The relationship between energy balance after calving and reproductive functions in

Holstein dairy cows treated by the OVSYNCH system. *Acta Univ. Agric. et Silvic. Mendel. Brun.*, 61, 3: 601–610. ISSN 1211-8516.

FILIPČÍK, R., HANULÁKOVÁ, Š., 2011: Vliv způsobu rozmrazení inseminační dávky skotu na aktivitu spermií, (The influence of the thawing method of a cattle insemination dose on sperm motility). *Výzkum v chovu skotu – Cattle Research*, 53, 3: 12–16. [in Czech]. ISSN 0139-7265.

HANULÁKOVÁ, Š., MAMICA, O., MÁCHAL, L., KŘIVÁNEK, I., FILIPČÍK, R., HOŠEK, M., CHLÁDEK, G., 2012: Physical properties of stallion semen in relation to some qualitative and quantitative characteristics. *Acta Univ. Agric. et Silvic. Mendel. Brun.*, 60, 6: 97–102. ISSN 1211-8516.

HANUŠ, O., FRELICH, J., TOMÁŠKA, M., VYLETĚLOVÁ, M., GENČUROVÁ, V., KUČERA, J., TŘINÁCTÝ, J., 2010: The analysis of relationships between chemical composition, physical, technological and health indicators and freezing point in raw cow milk. *Czech Journal of Animal Science*, 55: 11–29. ISSN 1212-1819.
HEGEDÜŠOVÁ, Z., ŠTOLC, L., LOUDA, F., ČUNÁT, L., VEJNAR, J., 2012: Effect of different extenders on ram sperm traits during storage. *Acta Univ. Agric. et Silvic. Mendel. Brun.*, 60, 6: 111–116. ISSN 1211-8516.

HORKÝ, P., JANČÍKOVÁ, P., ZEMAN, L., 2012: The effect of a supplement of chromium (picolinate) on the level of blood glucose, insulin activity and changes in laboratory evaluation of the ejaculate of breeding boars. *Acta Univ. Agric. et Silvic. Mendel. Brun.*, 60, 1: 49–56. ISSN 1211-8516.

JANETT, F., KEO, S., BOLLWEIN, H., HÄSSIG, M., THUN, R., 2005: Comparison of AndroMed®, Bioxcell® and Triladyl® extender for cryopreservation of bull semen. *Schweiz Archiv Tierheilk*, 147: 62–62. ISSN 0003-9438.

KAYA, A., AKSOY, M., TEKELI, T., 2002: Influence of ejaculation frequency on sperm characteristics, ionic composition and enzymatic activity of seminal plasma in rams. *Small Ruminant Research*, 44, 2: 153–158. ISSN 0921-4488.

LEBLANC, S., 2010: Assessing the Association of the Level of Milk Production with Reproductive Performance in Dairy Cattle. *Journal of Reproduction and Development*, 56: S1–S7. ISSN 0916-8818.

LOUDA, F., BJELKA, M., JEŽKOVÁ, A., POZDÍŠEK, J., STÁDNÍK, L., BEZDÍČEK, J., 2007: *Zásady využíváni plemenných býků v podmínkách přirozené plemenitby, (Principles of breeding bulls use on natural breeding conditions)*. 1. vyd. Rapotín: Research Institute for Cattle Breeding, Ltd, Rapotín, Czech Republic, 56 s. ISBN 978-80-87144-01-5 [in Czech].

MAURYA, V. P., TULI, R. K., 2003: Post-thaw thermal resistance test on motility and acrosomal integrity of filtered and non-filtered frozen semen of Murrah buffalo bulls. *Asian-Australasian Journal of Animal Sciences*, 16, 10: 1424–1428. ISSN 1011-2367.

MOUSSA, M., MARTINET, V., TRIMECHE, A., TAINTURIER, D., ANTON, M., 2002: Low density lipoproteins extracted from hen egg yolk by an easy method: cryoprotective effect on frozen-thawed bull semen. *Theriogenology*, 57, 6: 1695–1706. ISSN 0093-691X.

PACE, M. M., GRAHAM, E. F., 1974. Components in egg-yolk which protect bovine spermatozoa during freezing. *Journal of Animal Science*, 39, 6: 1144–1149. ISSN 1212-1819.

PADRIK, P., HALLAP, T., KAART, T., BULITKO, T., JAAKMA, U., 2012: Relationships between the results of hypo-osmotic swelling tests, sperm motility, and fertility in Estonian Holstein dairy bulls. *Czech Journal of Animal Science*, 57, 10: 490–497. ISSN 1212-1819.

ROCHE, J. R., BURKE, C. R., MEIER, S., WALKER, C. G., 2011: Nutrition x reproduction interaction in pasture-based systems: is nutrition a factor in reproductive failure? *Animal Production Science*, 51, 12: 1045–1066. ISSN 1836-0939.

SAS, 2011: SAS – STAT® 9.3. User's Guide. Carry, NC: SAS Institute Inc. 5121 s.

SIDDIQUE, M., ALI, R., RAZA, A., 2006: Effect of buffers on freezing of buffalo bull semen. *Journal of Agriculture & Social Sciences*, 2: 117–119. ISSN 1813-2235.

STRADAIOLI, G., NORO, T., SYLLA, L., MONACI, M., 2007: Decrease in gluthathione (GSH) content in bovine sperm after cryopreservation: comparison between two extenders. *Theriogenology*, 67, 7: 1249–1255. ISSN 0093-691X.

ŠTOLC, L., STÁDNÍK, L., JEŽKOVÁ, A., LOUDA, F., 2009: Relationships among herd, ram breeds, age of rams, sperm density before diluting and sperm motility during thermal survival test. *Acta Univ. Agric. et Silvic. Mendel. Brun.*, 57, 4: 109–116. ISSN 1211-8516.

THARA, K. M., NAIR, S. P., SURESH, 2007: Sire effect on *in vitro* fertilizability of matured cattle oocytes. *Indian Journal of Biotechnology*, 6, 3: 421–422. ISSN 0972-5849.

VÁGENKNECHTOVÁ, M., HOŠEK, M., MÁCHAL, L., CHLÁDEK, G., 2011: The influence of external and internal factors on the quality of semen collection and qualitative indicators of semen in the dog (*Canis familiaris*). *Acta Univ. Agric. et Silvic. Mendel. Brun.*, 59, 6: 373–380. ISSN 1211-8516.

WALSH, S.W., WILLIAMS, E.J., EVANS, A.C.O., 2011: A review of the causes of poor fertility in high milk producing dairy cows. *Animal Reproduction Science*, 123, 3–4: 127–138. ISSN 0378-4320.

ZINK, V., LASSEN, J., ŠTÍPKOVÁ, M., 2012: Genetic parameters for female fertility and milk production traits in first-parity Czech Holstein cows. *Czech Journal of Animal Science*, 57, 3: 108–114. ISSN 1212-1819.

Address

Ing. Jan Beran, Ph.D., Department of Animal Husbandry, Ing. Ondřej Šimoník, Department of Veterinary Sciences, Assoc. Prof. Ing. Luděk Stádník, Ph.D., Department of Animal Husbandry, Assoc. Prof. MVDr. Radko Rajmon, Ph.D., Department of Veterinary Sciences, Ing. Jaromír Ducháček, Ph.D., Department of Animal Husbandry, Ing. Adéla Krejcárková, Department of Veterinary Sciences, Ing. Martina Doležalová, Department of Animal Husbandry, Ing. Jiří Šichtař, Ph.D., Department of Veterinary Sciences, Faculty of Agrobiology, Food and Natural Resources, Czech University of Life Sciences Prague, Kamýcká 129, 165 21, Praha 6 – Suchdol, Czech Republic, e-mail: beranj@af.czu.cz

ACTA UNIVERSITATIS AGRICULTURAE ET SILVICULTURAE MENDELIANAE BRUNENSIS

Volume 64 44 Number 2, 2016
http://dx.doi.org/10.11118/actaun201664020395

EFFECT OF LDL ADDITION INTO SELECTED BULL SPERM DILUTERS ON RESISTANCE OF SPERMATOZOA AGAINST COLD SHOCK

Jan Beran[1], Ondřej Šimoník[2], Radko Rajmon[2], Luděk Stádník[1], Martina Doležalová[1], Adéla Krejcárková[2], Jaromír Ducháček[1], Jiří Šichtař[2]

[1] Department of Animal Husbandry, Faculty of Agrobiology, Food and Natural Resources, Czech University of Life Sciences Prague, Kamýcká 129, 165 21 Praha 6-Suchdol, Czech Republic
[2] Department of Veterinary Science, Faculty of Agrobiology, Food and Natural Resources, Czech University of Life Sciences Prague, Kamýcká 129, 165 21 Praha 6-Suchdol, Czech Republic

Abstract

BERAN JAN, ŠIMONÍK ONDŘEJ, RAJMON RADKO, STÁDNÍK LUDĚK, DOLEŽALOVÁ MARTINA, KREJCÁRKOVÁ ADÉLA, DUCHÁČEK JAROMÍR, ŠICHTAŘ JIŘÍ. 2016. Effect of LDL Addition Into Selected Bull Sperm Diluters on Resistance of Spermatozoa Against Cold Shock. *Acta Universitatis Agriculturae et Silviculturae Mendelianae Brunensis*, 64(2): 395–399.

The aim of work was to determine the effect of LDL cholesterol addition into selected diluters on the resistance of spermatozoa against cold shock and on their short-term survivability during cold test. The hypothesis was that the addition of LDL cholesterol will positively affects sperm resistance to cold shock and ensures a higher survivability of spermatozoa during short-term cold survival test. Four bulls of different breeds and ages, from the same sire insemination center were used. A total of eight semen collections were processed. Each ejaculate was divided into 6 portions (3 controls and 3 samples). Three commercially produced diluters, AndroMed®, Bioxcell®, and Triladyl® were used, each in standard and LDL enriched variants. In the case of AndroMed® or Bioxcell®, 6% of LDL was simply added. In Triladyl®, 10% of LDL replaced the standard egg yolk component. Spermatozoa resistance to cold shock was evaluated by the percentage of live sperm using Eosin-Nigrosine staining. The results showed the influence of bull individuality as an important factor. It is possible to recommend Bioxcell® with addition of LDL cholesterol in 6% concentration, which survivability was 69.17% at the beginning of the test, and 52.94% after 2 hours of incubation.

Keywords: AndroMed, Bioxcell, bull semen, LDL, Triladyl, sperm survivability

INTRODUCTION

During the processing of semen, spermatozoa are exposed to many non-physiological changes, e.g. of temperature, pH, osmotic pressure induced by cryoprotectants, formation and dissolution of ice crystals and of course the absence of female genital secretions, with which the sperm are mixed after ejaculation (Beran *et al.*, 2014). Biochemical and anatomical characteristics of sperm can be altered during the freezing process, plasma membrane is primarily affected (Hammerstedt *et al.*, 1990). So, the success of cryopreservation depends on many factors, including the interaction between cryoprotectants, type of diluters (Stádník *et al.*, 2015), speed of freezing/thawing (Doležalová *et al.*, 2015), packaging and individuality of donor (Cooter *et al.*, 2005; Clulow *et al.*, 2008).

Composition of diluters affects the viability and fertilization ability of sperm in the insemination dose (Siddique *et al.*, 2006). Therefore, the most suitable protecting media are continuously looking for and developing.

Egg yolk improves sperm function and keeps their fertilization ability (Barak *et al.*, 1992). The phospholipids and low-density lipoprotein (LDL) permeating into membranes are basic fractions of egg yolk which provide protection for sperm during cooling and freezing (Medeiros *et al.*, 2002). Egg yolk can be replaced in diluters by plant phospholipids due to its inconsistent composition (Ansari

et al., 2010) and risk of bacterial contaminations (Bousseau *et al.*, 1998). There is an assumption that LDL addition to commercially manufactured diluters (animal and/or plant phospholipid-based) can improve cryoprotective properties of them.

The potential fertility of sperm can be evaluated by several methods (Dhurvey *et al.*, 2012). Cold shock test was evaluated in relation to LDL addition to diluters (Beran *et al.*, 2013), but optimal LDL concentration increasing resistance of spermatozoa against cold shock was not suggested. Thus, the aim of this study was to determine the effect of the LDL addition to selected diluters on the rate of spermatozoa survival following cold shock and propose the optimal combination of the diluter and LDL concentration.

MATERIALS AND METHODS

Collection of Semen, Dilution and Processing

Three commercially available diluters – AndroMed®, Triladyl® (both from MiniTüb GmbH, Tiefenbach, Germany) and Bioxcell® (IMV, L' Aigle, France) were used. Control variants (CA, CB, and CT) of diluters were prepared according to the manufacturer's instructions. Experimental variants included 6% LDL cholesterol addition into the AndroMed® (A6) and Bioxcell® (B6) diluters, and 10% LDL addition into the Triladyl® (T10) diluter prepared without using egg yolk, which is normally its essential component. Increased concentration of LDL in Triladyl® replaced cryoprotective properties of egg yolk. The diluters used were prepared on the day of sampling and stored at the cooling box (4 °C).

Four bulls (bull A, B, C, and D) of different breeds (two bulls of Holstein breed, two bulls of Czech Fleckvieh breed), of ages from two to four years and the same frequency of collecting (once weekly) belonging to one sire insemination center were used. The bulls were proved and actively used for sperm production. Eight samples of ejaculate were obtained from selected bulls using an artificial vagina during the period of eight weeks. The samples of semen were then transferred to the university laboratory within one hour at 4 °C for next processing.

Samples of semen were pipetted using a sterile pipette to a sterile chilled (4 °C) tubes. Each sample of semen was immediately diluted to 50 000 spermatozoa/ml. The required amount of diluters was applied using sterile syringes directly to the tubes with samples. Thereafter tubes were sealed with sterile stoppers, mixed gently and placed into the cooling box (4 °C).

Evaluation of Resistance Against Cold Shock

Three capillaries (0.1 ml) were gradually filled from each sample at 4 °C, closed at one end with plasticine and stored for ten minutes in a cooled bath (No Ice, Bibby Scientific, Ltd., Staffordshire, UK) at 0 °C. After the end of incubation, Eosin-Nigrosine staining was done: the capillaries content was gently mixed with 20 µl of Eosin by circular motion for 30 sec. Then 40 µl of Nigrosine was added, gently mixed and smear was done from this suspension. This procedure was repeated after 2 hours incubation of extended semen samples in a water bath at 37 °C. Smears (36 of each collecting day, 288 together) were examined after drying under a phase contrast microscope (Eclipse E200, Nikon®, Tokyo, Japan) at 1 000× magnification and with oil immersion by one evaluator. Minimum of 100 spermatozoa was classified as either dead (with red heads) or live (with white heads) and expressed as a percentage rate of live sperm.

Statistical Analysis

The data set was analyzed using a generalized linear model in the statistical program SAS/STAT 9.1. (SAS Institute Inc., Cary, NC, USA). The following equation was used:

$$Y_{ijk} = \mu + BULL_i + SAMPLE_j + e_{ijk},$$

where

Y_{ijk} ..... observed value of the dependent variable (percentage rate of live sperm at the beginning and the end of 2 hours incubation, difference between these measurements),

μ ..... average value of the dependent variable,

$BULL_i$ ..... fixed effect of the i^{th} bull (i = bull A, n = 3; bull B, n = 3; bull C, n = 1; bull D, n = 1),

$SAMPLE_j$ ... fixed associated effect of the j^{th} sample of diluter and LDL addition combination (j = A6, n = 48; B6, n = 48; T10, n = 48; CA, n = 48; CB, n = 48; CT, n = 48),

e_{ijk} ..... residual effects.

The differences between the variables estimated were tested at the levels of significance $P < 0.05$ and $P < 0.01$.

RESULTS

The basic characteristics of the model equation used are presented in Tab. I. The coefficient of determination ranged from $r^2 = 0.24$ to 0.84. The sire individuality had a significant effect ($P < 0.01$) on all investigated characteristics (ACT 0, ACT 2 and ACT 0–ACT 2). The effect of sample (diluter and LDL addition) was statistically higher significant ($P < 0.01$) only on sperm survivability after 2 hours of the test (ACT 2) and on the difference between sperm survivability in time 0 and 2 hours of the test (ACT 0–ACT 2).

The results of evaluation of the effect of the bull on the sperm survivability are presented in Tab. II. The highest survivability of sperm at the beginning of the test was found in bull A (81.62%), the lowest was detected in bull D (36.44%, $P < 0.01$). The highest sperm survivability after 2 hours of the test duration was found in bull C (61.25%, $P < 0.05$–0.01), whereas the lowest was detected in bull D

I: *Basic characteristics of the model equation used for data analysis*

TRAIT	MODEL		BULL		SAMPLE	
	r^2	P	F-test	P	F-test	P
ACT 0	0.84	< 0.01	598.33	< 0.01	1.58	0.0813
ACT 2	0.56	< 0.01	123.48	< 0.01	4.18	< 0.01
ACT 0–ACT 2	0.24	< 0.01	21.97	< 0.01	2.94	0.0003

ACT 0 = sperm survivability at the beginning of the test; ACT 2 = sperm survivability after 2 hours of the test duration; ACT 0–ACT 2 = difference between sperm survivability in time 0 and 2 hours of the test.

II: *The effect of bull on sperm survivability*

	Bull A	Bull B	Bull C	Bull D
	LSM ± SE	LSM ± SE	LSM ± SE	LSM ± SE
ACT 0	81.62 ± 0.56^{CD}	80.61 ± 0.56^{CD}	70.34 ± 0.98^{ABD}	36.44 ± 0.98^{ABC}
ACT 2	57.43 ± 1.05^{D}	56.47 ± 1.05^{cD}	61.25 ± 1.82^{bD}	20.31 ± 1.82^{ABC}
ACT 0–ACT 2	24.18 ± 1.06^{CD}	24.14 ± 1.06^{CD}	9.09 ± 1.84^{ABD}	26.12 ± 1.84^{ABC}

ACT 0 = sperm survivability at the beginning of the test in percent; ACT 2 = sperm survivability after 2 hours of the test duration in percent; ACT 0–ACT 2 = difference between sperm survivability in time 0 and 2 hours of the test in percent; upper script letters means significant difference among sires – a, b, c, d = $P < 0.05$; A, B, C, D = $P < 0.01$.

III: *The effect of the diluter and LDL addition on sperm survivability against cold shock*

Sample	Label	ACT 0	ACT 2	ACT 0-ACT 2
		LSM ± SE	LSM ± SE	LSM ± SE
A6	A	67.86 ± 1.36	47.12 ± 2.52	20.74 ± 2.56^{D}
B6	B	69.17 ± 1.36^{e}	52.94 ± 2.52^{e}	16.23 ± 2.56^{e}
T10	C	65.97 ± 1.36	44.53 ± 2.52^{f}	21.44 ± 2.56^{f}
CA	D	64.75 ± 1.36	47.85 ± 2.52^{E}	16.90 ± 2.56^{AE}
CB	E	65.61 ± 1.36^{b}	46.16 ± 2.52^{bD}	19.46 ± 2.56^{bD}
CT	F	67.41 ± 1.36	51.40 ± 2.52^{c}	16.01 ± 2.56^{c}

ACT 0 = sperm survivability at the beginning of the test in percent; ACT 2 = sperm survivability after 2 hours of the test duration in percent; ACT 0–ACT 2 = difference between sperm survivability in time 0 and 2 hours of the test in percent; A6 = AndroMed® with 6% LDL; B6 = Bioxcell® with 6% LDL; T6 = Triladyl® with 10% LDL; CA = control samples of AndroMed®; CB = control samples of Bioxcell®; CT = control samples of Triladyl®; upper script letters means significant difference among samples – a, b, c, d, e, f = $P < 0.05$; A, B, C, D, E, F = $P < 0.01$.

again (20.31%, $P < 0.01$). The smallest difference in sperm survivability (9.09%, $P < 0.01$) was reached by bull C, on the other hand the highest difference was determined in bull D (26.12%, $P < 0.01$). Results presented in Tab. II documented significant variability of sperm survival among selected sires at the beginning of observation, at the end of the test, as well as in sperm survival decline during the entire test performed.

The results of evaluation of the diluter and LDL addition effect on sperm survivability against cold shock are presented in Tab. III. Significantly the highest survivability of sperm at the beginning of the test was found in sample B6 (69.17%) compared to CB (65.61%; $P < 0.05$), and the lowest was detected in sample CA (64.75%; $P > 0.05$). Significantly the highest sperm survivability after 2 hours of the test duration was found again in sample B6 (52.94%) in comparison with CB (46.16%; $P < 0.05$) and significantly the lowest values were detected in sample T10 (44.53%) compared to CT (51.40%; $P < 0.05$). Some of other differences among evaluated diluters at the end of the test were significant as well ($P < 0.05$–0.01). The difference between the beginning and the end of the test duration was the largest in sample T10 (21.44%) and the lowest were detected in CT (16.01%; $P < 0.05$). The second lowest decline of sperm survival was detected in sample B6 (16.23%), significantly ($P < 0.05$) different from CB (19.46%). Some of measured differences of sperm survival decline were statistically significant to each other as well ($P < 0.05$–0.01).

DISCUSSION

The experiment brought some interesting results. Using the extracted LDL – cholesterol from egg yolk has been investigated previously in various extenders (Vera-Munoz *et al.*, 2009), at different concentrations (Moussa *et al.*, 2002) and confirmed by thermal test or cryoconservation, but the cold shock test was used in this experiment for the first time. According to Anton *et al.* (2003) is known that egg yolk has a positive effect during sperm cryopreservation, because the components in egg yolk can create the absorption of oil and

water phase interface and make the oil droplets protective film. These abilities include membrane stabilization or cool shock protection. Egg yolk contains phospholipids; the largest amount is represented by LDL – cholesterol, which provides sperm cryoprotection during freezing or thawing by increasing the stability of the plasma membrane (Moussa *et al.*, 2002). Watson (1995) stated that is important to test both, the effect of heat and cold on sperm survivability. Chantler *et al.* (2000) confirmed the loss of motility of sperm if the cryoprotective substances (e.g. egg yolk) were not added into ejaculate. Cold shock was also investigated in boar sperm survivability using egg yolk. Positive results attributed to the composition of egg yolk, natural source of LDL cholesterol (Hu *et al.*, 2006).

Individuality of bulls has very important effect on sperm survivability. Effect of bull was significant ($P < 0.01$) on all monitored indicators. The significantly ($P < 0.01$) lowest sperm survivability was found in bull D in all observed characteristics. On the other hand the highest sperm survivability had bull A at the beginning of the cold test and bull C at the end of the test; bull C had simultaneously the lowest ($P < 0.01$) difference between the beginning and the end of the test. In general we can say that considerable individual differences in sperm survivability between bulls were determined. This is in accordance with the work of Beran *et al.* (2012 and 2013).

We can assume that the associated effect of diluter and LDL addition on sperm survivability against cold shock was highly significant ($P < 0.01$) only after 2 hours of the test. This is in accordance with Vera-Munoz *et al.* (2011) findings. They confirmed that samples with LDL showed better sperm motility and plasma membrane integrity even after 8 days of incubation. Similarly, Amirat *et al.* (2005) determined the lowest damage of sperm diluted with LDL after 4 hours incubation.

Based on our results, we can say that the samples of AndroMed® and Bioxcell® enriched by LDL achieved balanced or higher sperm survival (–0.73% to +6.78%) compared to control samples. Control Triladyl® contained 20% of egg yolk and provided higher level of sperm survival during the test (+1.44% and +6.87%) than 10% addition of LDL cholesterol as substitution of egg yolk. Concurrently, Bioxcell® with 6% LDL addition was the best variant for resistance of sperm following cold shock. This variant had the highest values of sperm survivability at the beginning (69.17%) and after 2 hours incubation (52.94%) compared to Triladyl® and AndroMed®. Bioxcell® with 6% LDL addition had the smallest decline during the entire test (16.23%) as well. However, significant differences were determined only to control variant of Bioxcell®. The combination of Bioxcell® and 6% of LDL was the most favorable for sperm due to the lower decline of motility compared to other experimental variants of diluters.

If we compare the control samples each other we can state that the best variant is the diluter Triladyl®. This variant has achieved the lowest decline (16.01%). This is in opposite with Vera-Munoz *et al.* (2009) who state that the LDL diluter had better results than Triladyl® and AndroMed®.

CONCLUSION

Generally, we can recommend addition of 6% LDL into diluter Bioxcell® according to significantly higher sperm survival and its lowest decline in comparison with control variant of Bioxcell®.

Acknowledgement

The paper was supported by „S" grant of MŠMT ČR and NAZV [No. QJ1210109].

REFERENCES

AMIRAT, L., ANTON, M., TAINTURIER, D., CHATAGNON, G., BATTUT, I., COURTENS, J. L. 2005. Modifications of bull spermatozoa induced by three extenders: Biociphos, low density lipoprotein and Triladyl, before, during and after freezing and thawing. *Reproduction*, 129(4): 535–543.

ANSARI, M. S., RAKHA, B. A., ANDRABI, S. M. H., AKHTER, S. 2010. Usefulness of powdered and fresh egg yolk for cryopreservation of Zebu bull spermatozoa. *Reprod. Biol.*, 10(3): 235–240.

ANTON, M., MARTINET, V., DALGALARRONDO, M., BEAURNAL, V., DAVID-BRIAND, E., RABESONA, H. 2003. Chemical and structural characterisation of low-density lipoproteins purified from hen egg yolk. *Food Chem.*, 83(2): 175–183.

BARAK, Y., AMIT, A., LESSING, J. B., PAZ, G., HOMMONAI, Z. T., YOGEV, L. 1992. Improved fertilization rate in an in vitro fertilization program by egg yolk-treated sperm. *Fertil. Steril.*, 58: 197–198.

BERAN, J, STÁDNÍK, L., BEZDÍČEK, J., LOUDA, F., ČÍTEK, J., DUCHÁČEK, J. 2012. Effect of sire and extender on sperm motility and share of live or dead sperm in bulls' fresh ejaculate and in AI doses after thawing. *Arch. Tierz.-Arch. Anim. Breed.*, 55(3): 207–218.

BERAN, J., ŠIMONÍK, O., STÁDNÍK, L., RAJMON, R., DUCHÁČEK, J., KREJCÁRKOVÁ, A., DOLEŽALOVÁ, M., ŠICHTAŘ, J. 2013. Effect of bull, diluter and LDL-cholesterol concentration on spermatozoa resistance against cold shock. *Acta Univ. Agric. Silvic. Mendel. Brun.*, 61(6): 1575–1581.

BERAN, J., STÁDNÍK, L., DOLEŽALOVÁ, M., TOUŠOVÁ, R. 2014. *Improving the quality of bulls insemination doses by selecting a suitable semen extender* [in Czech: *Zlepšení kvality inseminačních dávek býků výběrem vhodného ředidla ejakulátu*]. 1st ed. Prague: Czech University of Life Sciences Prague

BOUSSEAU, S., BRILLARD, J. P., MARQUANT-LE GUIENNE, B., GUERIN, B., CAMUS, A., LECHAT, M. 1998. Comparison of bacteriological qualities of various egg yolk sources and the in vitro and in vivo fertilizing potential of bovine semen frozen in egg yolk or lecithin based diluents. *Theriogenology*, 50(5): 699–706.

CHANTLER, E., ABRAHAM-PESKIR, J. V., LITTLE, S., MCCANN, C., MEDENWALDT, R. 2000. Effect of cooling on the motility and function of human spermatozoa. *Cryobiology*, 41(2): 125–134.

CLULOW, J. R., MANSFIELD, L. J., MORRIS, L. H. A., EVANS, G., MAXWELL, W. M. C. 2008. A comparison between freezing methods for the cryopreservation of stallion spermatozoa. *Anim. Reprod. Sci.*, 108(3–4): 298–308.

COOTER, P. Z., GOOLSBY, H. A., PRIEN, S. D. 2005. Preliminary evaluation of a unique freezing technology for bovine spermatozoa cryopreservation. *Reprod. Dom. Anim.*, 40(2): 98–99.

DHURVEY, M., GUPTA, V., NEMA, S., PATIDAR, A., SHIVHARE, M., SINGHT, N., SHAKYA, V. 2012. Modern semen evaluation techniques in domestic animals: A review. *DHR-IJBLS*, 3(1): 62–83.

DOLEŽALOVÁ, M., STÁDNÍK, L., BINIOVÁ, Z., DUCHÁČEK, J., BERAN, J. 2015. Effect of freezing curve type on bull spermatozoa motility after thawing. *Acta Vet. Brno*, 84(4): 383–391.

HAMMERSTEDT, R. H., GRAHAM, J. K., NOLAN, J. P. 1990. Cryopreservation of mammalian sperm – what ask them to survive. *J. Androl.*, 11: 73–88.

HU, J. H., LI, Q. W., GANG, L., CHEN, X. Y., HAI, Y., ZHANG, S. S., WANG, L. Q. 2006. The cryoprotective effect on frozen-thawed boar semen of egg yolk low density lipoproteins. *Asian Australas. J. Anim. Sci.*, 19(4): 486–494.

MEDEIROS, C. M. O., FORELL, F., OLIVEIRA, A. T. D., RODRIGUES, J. L. 2002. Current status of sperm cryopreservation: why isn't it better? *Theriogenology*, 57(1): 327–344.

MOUSSA, M., MARTINET, V., TRIMECHE, A., TAINTURIER, D., ANTON, M. 2002. Low density lipoproteins extracted from hen egg yolk by an easy method: cryoprotective effect on frozen-thawed bull semen. *Theriogenology*, 57(6): 1695–1706.

SIDDIQUE, M., ALI, R., RAZA, A. 2006. Effect of buffers on freezing of buffalo bull semen. *J. Agric. Soc. Sci.*, 2(2): 117–119.

STÁDNÍK, L., RAJMON, R., BERAN, J., ŠIMONÍK, O., DOLEŽALOVÁ, M., ŠICHTAŘ, J., STUPKA, R., FOLKOVÁ, P. 2015. Influence of selected factors on bovine spermatozoa cold shock resistance. *Acta Vet. Brno*, 84(2): 125–131.

VERA-MUNOZ, O., AMIRAT-BRIAND, L., DIAZ, T., VASQUEZ, L., SCHMIDT, E., DESHERCES, S., ANTON, M., BENCHARIF, D., TAINTURIER, D. 2009. Effect of semen dilution to low-sperm number per dose on motility and functionality of cryopreserved bovine spermatozoa using low-density lipoproteins (LDL) extender: Comparison to Triladyl (R) and Bioxcell (R). *Theriogenology*, 71(6): 895–900.

VERA-MUNOZ, O., AMIRAT-BRIAND, L., BENCHARIF, D., ANTON, M., DESHERCES, S., SHMITT, E., THORIN, C., TAINTURIER, D. 2011. Effect of low-density lipoproteins, spermatozoa concentration and glycerol on functional and motility parameters of bull spermatozoa during storage at 4 degrees C. *Asian J. Androl.*, 13(2): 281–286.

WATSON, P. F. 1995. Recent developments and concepts in the cryopreservation of spermatozoa and the assessment of their post-thawing function. *Reprod. Fertil. Dev.*, 7(4): 871–891.

Contact information

Jan Beran: h.beran@seznam.cz
Ondřej Šimoník: simoniko@af.czu.cz
Radko Rajmon: rajmon@af.czu.cz
Luděk Stádník: stadnik@af.czu.cz
Martina Doležalová: dolezalovamartina@af.czu.cz
Adéla Krejcárková: krejcarkova@af.czu.cz
Jaromír Ducháček: duchacek@af.czu.cz
Jiří Šichtař: sichtar@af.czu.cz

4.3 Možnost zvýšení kryoprotektivních vlastností ředidel bez obsahu vaječného žloutku - vliv adice LDL do ředidel na bázi sójového lecitinu na funkční parametry spermií býků po rozmrazení

I přes dlouhodobou snahu výzkumných týmů zvýšit efektivitu kryokonzervace spermií býků je v současnosti uváděno jak vědeckými zdroji (Layek et al., 2016), tak i z praxe (MVDr. Koubková CRV Czech Republic spol s.r.o. 2017, pers. comm.), že až 50 % buněk je nenávratně během procesu kryokonzervace poškozeno. Jednou z hlavních strategií, jak zvýšit procento intaktních spermií po rozmrazení, a tím kvalitu inseminačních dávek, je modifikace receptury ředidel semene, především co se týče obsažených kryoprotektantů (Moce et al., 2010a). Široce využívána jsou ředidla s obsahem vaječného žloutku (Celeghini et al., 2008), ovšem přítomnost žloutku představuje určité překážky a rizika (Amirat et al., 2004; Amirat et al., 2005). U ředidel bez obsahu komponent živočišného původu, kde je obsažen sójový lecitin nebyla prozatím jednoznačně prokázána srovnatelná kryoprotektivní účinnost v porovnání s prvně jmenovanými (Crespilho et al., 2012; Murphy et al., 2017). Pro zachování životaschopnosti a tím i fertilizační schopnosti spermií je zásadní zachování integrity plazmatické membrány. Z tohoto hlediska je velice podstatný cholesterol, přičemž LDL, jako hlavní komponenta vaječného žloutku podmiňující jeho kryoprotektivní vlastnosti, ve své struktuře cholesterol nese, spolu s další důležitou složkou, kterou jsou antioxidanty (Anton et al., 2003; Harisa et Alanazi, 2014). Je tedy otázkou, zda by LDL mohlo přispět ke zvýšení kryoprotektivních vlastností ředidel s obsahem sójového lecitinu.

Vzhledem k tomu, že LDL není komerčně dostupné, jeho izolace probíhala na základě publikované metodiky (Moussa et al., 2002). Pro účely dlouhodobějšího uchovávání LDL byl metodický postup ve finálních fázích modifikován přidáním konzervantu azidu sodného. Námi izolovaný LDL bylo tedy nutné otestovat ve smyslu porovnatelnosti s výsledky ostatních autorů.

Hypotéza: Modifikace receptury vybraných ředidel na bázi sójového lecitinu pomocí LDL povede ke zvýšení jejich kryoprotektivních vlastností.

Cíl: Stanovit efekt adice LDL do ředidel na bázi sójového lecitinu na funkční parametry spermií býků po kryokonzervaci.

V prvotní studii byla testována širší škála ředidel a koncentrací LDL. Vliv LDL na funkci spermií po rozmrazení byl hodnocen na základě průměrných hodnot kinematických parametrů motility spermií a jejich viability. Výsledky ukázaly pozitivní efekt 6% LDL v případě adice do

ředidla Bioxcell, tato koncentrace se tak ukázala ve spojení s tímto ředidlem jako potenciálně optimální.

V navazující studii byl hodnocen vliv adice LDL do ředidel na bázi sójového lecitinu již jen ve vybrané koncentraci 6%. Na základě zpracovaného review a praktických zkušeností ze zahraniční stáže byl použit alternativní statistický přístup k hodnocení motility spermií z CASA clusterovou analýzou. Tato multiparametrická statistická metoda zohledňuje biologickou podstatu vzorků tím, že poskytuje možnost hodnocení jednotlivých subpopulací spermií ve vzorku (Holt et al., 2007). Pozitivní vliv LDL na distribuci jednotlivých subpopulací spermií byl zřetelný u obou vybraných ředidel Bioxcell i Andromed. Podobně jako u motility se projevil pozitivní vliv LDL i na integritu akrozómu spermií. Signifikantní se ukázal v případě adice LDL do ředidla Bioxcell. Zajímavé je, že při hodnocení funkčního stavu mitochondrií se efekt LDL ukázal jako významný naopak u ředidla Andromed. Procento živých spermií po rozmrazení s intaktní plazmatickou membránou nebylo překvapivě přítomností LDL v žádném z ředidel podpořeno. Lze předpokládat, že rozdíly ve vlivu LDL mohou souviset s odlišným složením samotných ředidel, především co se týče procentuálního zastoupení a sójového lecitinu.

Výsledky studií ukázaly prokazatelný efekt adice LDL do ředidel s obsahem sójového lecitinu, kdy konkrétní dopad na spermie po rozmrazení souvisí s použitým ředidlem. Adicí LDL je tedy možné zvyšovat kryoprotektivních vlastnosti ředidel s obsahem sójového lecitinu.

Hypotéza: Substitucí vaječného žloutku v ředidle pomocí LDL ošetřeného azidem sodným bude dosaženo výsledků srovnatelných se standardy uváděnými v literatuře.

Cíl: Ověřit standardní účinnost LDL při substituci vaječného žloutku v ředidle a porovnat s publikovanými standardy s ohledem na použití azidu sodného pro jeho dlouhodobé uchovávání.

Z hlediska praktické využitelnosti bylo důležité ověřit standardní kvalitu a funkci námi produkovaného LDL. V rámci studie Simonik et al. (2016) byla testována i substituce vaječného žloutku v ředidlech pomocí LDL, přičemž výsledky byly shodné s výstupy studií ostatních autorů. Ukázala se tak standardní kvalita a funkce námi produkovaného LDL, které bylo v konečných fázích izolace přechodně konzervováno azidem sodným.

Byla tedy potvrzena možnost prodloužené skladovatelnosti LDL před jeho použitím. Tento fakt, jako další prvek novosti představuje důležitý krok z hlediska potenciálu praktického využití LDL a translace výsledků výzkumu do praxe.

Publikace:

Šimoník, O., Rajmon, R., Stádník, L., Šichtař, J., Beran, J., Ducháček, J., Hodek, P., Trefil, P. 2016. Effect of low-density lipoprotein addition to soybean lecitin-based extenders on bull spermatozoa following freezing-thawing – preliminary results. Czech Journal of Animal Science 61 (12) 560-567.

Simonik, O., Beran J., Manaskova-Poslerova, P., Sichtar, J., Stadnik, L., Tumova, L., Dolezalova, M., Folkova, P., Rajmon R. 2018. Low Density Lipoprotein – important player in increasing cryoprotective efficiency of soybean lecitin-based bull semen extenders. Reproduction in Domestic Animals (under review).

Original Paper *Czech J. Anim. Sci., 61, 2016 (12): 560–567*

doi: 10.17221/27/2016-CJAS

Effect of low-density lipoprotein addition to soybean lecithin-based extenders on bull spermatozoa following freezing-thawing – preliminary results

O. Šimoník[1], R. Rajmon[1], L. Stádník[2], J. Šichtař[1], J. Beran[2], J. Ducháček[2], P. Hodek[3], P. Trefil[4]

[1]Department of Veterinary Sciences, Faculty of Agrobiology, Food and Natural Resources, Czech University of Life Sciences Prague, Prague, Czech Republic
[2]Department of Animal Husbandry, Faculty of Agrobiology, Food and Natural Resources, Czech University of Life Sciences Prague, Prague, Czech Republic
[3]Department of Biochemistry, Faculty of Science, Charles University in Prague, Prague, Czech Republic
[4]BIOPHARM, Research Institute of Biopharmacy and Veterinary Drugs, Jílové u Prahy, Czech Republic

ABSTRACT: Soybean lecithin-based extenders represent an alternative to extenders containing egg yolk, but there has been discussion about their cryoprotective efficacy. Low-density lipoprotein (LDL) was proved as a cryoprotective agent, which can replace egg yolk in extenders. The aim of this study was to investigate the effects of LDL addition to the soybean extenders on their cryoprotective properties. The effect of the LDL of our production was verified using commercial egg yolk extender BULLXcell®, 6%, 8%, and 10% LDL (v/v) as an egg yolk replacement. The effects of LDL addition to the soybean lecithin-based extenders in concentrations of 4%, 6%, and 8% (v/v) were tested using extenders AndroMed® and Bioxcell®. In total, 64 samples from eight bulls were evaluated. Kinematic parameters of spermatozoa, resulting from Computer Assisted Sperm Analysis, and their viability, evaluated by fluorescent technique, were assessed immediately after thawing and after 2 hours. The quality of LDL compared to other studies was confirmed, and the beneficial effects of egg yolk replacement by LDL were proved in extender BULLXcell®. 8% LDL provided the best values for the majority of kinematic parameters ($P < 0.05$), without effect on total motility ($P > 0.05$). Furthermore, addition of 4%, 6%, and 8% LDL to the soybean lecithin-based extender Bioxcell® showed a positive effect on the majority of kinematic parameters of spermatozoa ($P < 0.05$) at both times of incubation. However there was no significant influence on total motility ($P > 0.05$). Viability was higher after thawing in the case of 8% LDL ($P < 0.05$). However, there was no consistent effect of LDL addition to the AndroMed® extender. In conclusion, cryoprotective properties of the semen extenders based on a soybean lecithin can be improved by the addition of LDL.

Keywords: bull sperm; cryopreservation; sperm motility; viability; cryoprotectant

INTRODUCTION

Artificial insemination (AI) is a reproductive biotechnology method that has been intensively used worldwide, especially in dairy cattle breeding. For AI in cattle breeding, cryopreserved insemination doses (ID) are used (Zhang et al. 2015) and, consequently, their quality plays an important role

Supported by the Ministry of Education, Youth and Sports of the Czech Republic ("S" grant), by the Ministry of Agriculture of the Czech Republic (Project No. QJ1210109), and by the Grant Agency of the Charles University in Prague (Project UNCE 204025/2012).

doi: 10.17221/27/2016-CJAS

in the success of fertilization (Beran et al. 2013). However, cryopreservation represents a limiting factor in several of the advantages of AI due to its impacts on structural integrity and the physiological processes of spermatozoa (Amirat-Briand et al. 2009; Dzyuba et al. 2015; Sieme et al. 2015; Meamar et al. 2016).

To ensure spermatozoa protection against harmful effects of cryopreservation, such as the "cold shock" (Drobnis et al. 1993; Stadnik et al. 2015), crystal formation (Koshimoto et al. 2000) or reactive oxygen species generation (Alomar et al. 2016), the collected semen has to be diluted with a suitable freezing extender. As many authors have demonstrated, optimization of extender composition can have a positive effect on the undesirable changes in spermatozoa caused by cryopreservation (Spalekova et al. 2014; Muchlisin et al. 2015). Egg yolk and glycerol are the cryoprotectants that are most often utilized in the field of bovine semen cryopreservation (Aires et al. 2003). However, there have been efforts to replace the egg yolk in extenders by another substance due to possible risks connected with bacteria or mycoplasma contamination and for standardization of the extender composition (Crespilho et al. 2012). Additionally, egg yolk contains detrimental constituents, such as high-density lipoprotein or granules, which can inhibit physiological processes in the spermatozoa (Wall and Foote 1999).

Soybean lecithin, which generally consists of phosphatidylcholine and a mixture of fatty acids, is an alternative to egg yolk (Vishwanath and Shannon 2000). However, studies evaluating the efficiency of bovine semen extenders based on soybean lecithin or egg yolk have shown varied results, in favour of the latter type of extender (e.g. Gil et al. 2000; van Wagtendonk-de Leeuw et al. 2000; Thun et al. 2002; Aires et al. 2003; Beran et al. 2012; Crespilho et al. 2012). Another alternative to egg yolk in extenders is its substitution only by the compound responsible for its cryoprotective properties. In 1974 it was discovered that this compound is the major fraction of egg yolk plasma, i.e. low-density lipoprotein or LDL (Pace and Graham 1974). Different cryoprotective properties of LDL, that positively and directly affected spermatozoa structure (Bergeron et al. 2004) and/or extracellular conditions, were found (Hu et al. 2011). The positive effects of egg yolk substitution by LDL in semen extenders on qualitative parameters of spermatozoa have been repeatedly demonstrated (Moussa et al. 2002; Vera-Munoz et al. 2009; Amirat-Briand et al. 2010; Hu et al. 2010, 2011). The question then arises on whether the cryoprotective properties of soybean lecithin-based extenders could be improved by LDL addition. Furthermore, none of the above-mentioned studies describes any form of LDL preservation, which limits the practical use of LDL. However, the shelf-life of produced LDL may be stabilized and prolonged using sodium azide.

Therefore, the aim of this study was to investigate the effects of LDL addition to the soybean extenders on their cryoprotective properties.

MATERIAL AND METHODS

LDL extraction. Low-density lipoprotein with 97% purity was prepared according to the methodology of Moussa et al. (2002). Hen eggs were obtained from the controlled breeding program of BIOPHARM Czech Republic. In comparison to the methodology established by Moussa et al. (2002), there were slight modifications in our study. Sodium azide (0.1%) was used for preservation of the produced LDL and to minimize sanitary risks. Before using the LDL, sodium azide was removed by extensive dialysis against phosphate buffer saline (PBS).

Preparation of the extenders. Extenders were prepared at the beginning of the experiments. To verify the quality of LDL, the egg yolk extender BULLXcell® was used (IMV Technologies, L'Aigle, France). The standard composition according to the producer's manual with egg yolk served as a control, and in experimental variants, the egg yolk was substituted by 6%, 8%, and 10% LDL (v/v).

Soybean lecithin-based extenders, AndroMed® (Minitübe, Tiefenbach, Germany) and Bioxcell® (IMV Technologies) were used for testing the effect of LDL addition. These media were prepared in accordance with the manufacturer's instructions, and then LDL was added in concentrations of 4%, 6% or 8% (v/v) to each of the aforementioned extenders. Extenders without LDL were used as a control.

Collection and processing of semen. The semen was collected from eight bulls in a standard way at the insemination centre (Natural Ltd., Hradišťko pod Medníkem, Czech Republic). Each ejaculate was submitted to the basic assessment done by

doi: 10.17221/27/2016-CJAS

trained laboratory technician from the insemination centre. The following parameters were evaluated: ejaculate volume, sperm concentration, and percentage of motile sperm. Only ejaculates that conformed to the limits of sperm concentration (≥ 0.7×10^9/ml) and percentage of motile spermatozoa (≥ 70%) were used in this study. Semen was divided into equal fractions in relation to the number of tested variants and diluted to a final concentration of 120×10^6 spermatozoa/ml. Diluted semen was placed into polyvinyl straws (0.25 ml) and equilibrated at 5°C for 2 h. After this period, straws were cryopreserved using a computerized freezing machine (DigitCool®; IMV Technologies) with the standard freezing curve for bovine semen and then immersed directly into liquid nitrogen (–196°C) for storage. Straws were analyzed at least one week after the cryopreservation. Before each evaluation, the straws were thawed in the standard way in water bath (37°C/30 s). All analyses were performed 10 min after thawing ("incubation 0 h") and after a 2-hour thermoresistance test ("incubation 2 h") at the same temperature 37°C.

Evaluation of sperm motility. Sperm motility was assessed with the Computer Assisted Sperm Analysis (CASA) module NIS Elements Ar 4.20 (Laboratory Imaging Ltd., Prague, Czech Republic), using a DMK 23UM021 camera (The Imaging Source Europe GmbH, Bremen, Germany) with a frame rate of 60 images/s and a stereo microscope Nikon Eclipse E600 (Nikon Corp., Tokyo, Japan) with a heated plate. Thawed samples were transferred into the 1.5 ml plastic microtubes and diluted with physiological saline (pH 6.8) to a final concentration of $20–40 \times 10^6$ spermatozoa/ml, according to Verstegen et al. (2002). Next, 3 µl of the sample were evaluated in a calibrated Leja® counting chamber (Leja, Nieuw-Vennep, The Netherlands) with a depth of 20 µm in six different fields per sample. At least 200 trajectories per field were analyzed. The parameter total motility (TM) was evaluated on the basis of threshold of motile spermatozoa VAP > 20 µm/s. Selected kinematic parameters were analyzed: curvilinear velocity (VCL, µm/s), velocity of average path (VAP, µm/s), straight line velocity (VSL, µm/s), linearity (LIN, %), and amplitude of lateral head displacement (ALH, µm). For each sample, a total of 41 frames were captured at aforementioned frequency of camera (60 frames/s).

Evaluation of sperm viability. For the evaluation of sperm viability, a fluorescent technique according to Harrison and Vickers (1990) was used. Briefly, sperm samples were diluted with physiological saline (ph 6.8) to 950 µl to a final concentration of $1–10 \times 10^6$ spermatozoa/ml. This suspension was further supplemented with 20 µl of 5(6) carboxyfluorescein diacetate (CFDA) (Sigma Aldrich, St. Louis, USA) working solution (0.46 mg CFDA/ml dimethyl sulfoxide), 20 µl propidium iodide (PI) (Sigma Aldrich) working solution (0.5 mg PI/ml physiological saline), and 10 µl of 0.3% formaldehyde. The samples were then incubated in the dark at 37°C for 10 min. After incubation, 7 µl of the sample was transferred to a microscopic slide and mounted under a coverslip using nail polish. Evaluation was performed using a fluorescent microscope Nikon Eclipse E6000 (Nikon Corp.) at 400× magnification. Live spermatozoa with functional esterases emitted green fluorescence, while dead spermatozoa were red due to the intercalation of propidium iodide. For each sample, three replicates were evaluated, which means that 600 spermatozoa were counted per sample.

Statistical analysis. The dataset was evaluated using the SAS software (Statistical Analysis System, Version 9.3, 2011). To evaluate the indicators, a relevant model was selected using the REGG procedure and STEPWISE method. The effect of LDL concentration in each extender was assessed separately for incubation. The effect of bull in regression was added into the equation. The differences between samples and groups were evaluated using the GLM procedure and the Tukey-Cramer test.

Model equation. The following model was applied:

$$y_{ij} = \mu + a_i + b^*(BULL) + e_{ij}$$

where:

- y_{ij} = dependent variable (amplitude of lateral head displacement, linearity, velocity of average path, curvilinear velocity, straight line velocity)
- μ = mean value of dependent variable
- a_i = fixed effect of LDL concentration (BULLXcell®: $i = 0$, $n = 5225$; $i = 6$, $n = 6321$; $i = 8$, $n = 6118$; $i = 10$, $n = 6420$; Bioxcell®: $i = 0$, $n = 5696$; $i = 4$, $n = 4637$; $i = 6$, $n = 5434$; $i = 8$, $n = 5213$; AndroMed®: $i = 0$, $n = 6202$; $i = 4$, $n = 7116$; $i = 6$, $n = 6834$; $i = 8$, $n = 6581$)
- b*(BULL) = linear regression for bulls
- e_{ij} = random error

The significance was evaluated at the level $P < 0.05$.

doi: 10.17221/27/2016-CJAS

RESULTS

Effect of egg yolk replacement with LDL on motility and viability of spermatozoa. After thawing (T0) and 2 h of incubation (T2) there was no significant influence of LDL on TM ($P > 0.05$) (Table 1). The influence of egg yolk substitution with various concentration of LDL in the BULLXcell® extender on sperm motility and viability at T0 and T2 is shown in Table 1. All the chosen concentrations of LDL (6%, 8%, and 10% v/v) significantly influenced the kinematic parameters of motility at T0 as well as at T2 ($P < 0.05$). Substitution of egg yolk with particular concentrations of LDL in the BULLXcell® extender significantly increased the values of VAP, VCL, and VSL at T0 and T2 ($P < 0.05$). Viability of sperm was negatively influenced at T0 ($P < 0.05$) when egg yolk was substituted with 6% LDL; however, this effect was not observed after 2 h of incubation (T2).

Effect of LDL addition to soybean extenders on motility and viability of spermatozoa. There were no significant differences ($P > 0.05$) in TM between concentrations of LDL added to extender Bioxcell®. There was a significant positive effect ($P < 0.05$) of all added LDL concentrations on VAP, VCL, VSL, and ALH of spermatozoa treated with Bioxcell® extender at T0 (Table 2). These kinematic parameters were significantly higher ($P < 0.05$) when 4%, 6%, and 8% LDL (v/v) was added to Bioxcell® compared to the control. The same effect was seen after 2 h of incubation (T2) in VAP, VCL, and VSL ($P < 0.05$). Significant differences ($P < 0.05$) between individual concentrations of LDL were seen at T0 and T2. Addition of 8% LDL significantly ($P < 0.05$) increased the percentage of viable spermatozoa compared to the Bioxcell® control at T0. However, the proportion of viable spermatozoa after 2 h of incubation was the same for Bioxcell® with and without the addition of LDL ($P > 0.05$).

The effect of various concentrations of LDL on TM was also insignificant ($P > 0.05$), however the influence on evaluated kinematic parameters of spermatozoa was not as distinct in AndroMed® as in the case of Bioxcell® (Table 3). The effect of LDL (4%, 6%, and 8% v/v) addition to the AndroMed® extender was not beneficial, although differences between individual concentrations in ALH, LIN, VCL, and VSL were significant ($P < 0.05$) at T0. After 2 h of incubation (T2), spermatozoa in AndroMed® containing 8% LDL demonstrated significantly higher ($P < 0.05$) values of ALH, LIN, VAP, VCL, and VSL parameters compared to the AndroMed® control without LDL. A significantly higher ($P < 0.05$) number of viable spermatozoa were present at T0 in samples containing 4% LDL compared to the AndroMed® control.

Table 1. Effect of egg yolk replacement by low-density lipoprotein (LDL) on motility and viability of frozen-thawed spermatozoa

		Viability	TM	ALH	LIN	VAP	VCL	VSL
Incubation 0 h								
BULLXcell®		51.23 ± 2.32^{A}	56.71 ± 5.12	4.96 ± 0.06^{a}	44.11 ± 0.34^{a}	57.81 ± 0.88^{a}	107.67 ± 1.72^{a}	50.76 ± 0.87^{a}
	6%	41.57 ± 2.08^{B}	50.46 ± 4.67	5.32 ± 0.06^{b}	37.91 ± 0.31^{b}	64.42 ± 0.88^{b}	135.68 ± 1.60^{b}	54.94 ± 0.81^{b}
LDL	8%	50.86 ± 2.131	54.20 ± 4.33	5.81 ± 0.05^{c}	39.01 ± 0.29^{b}	73.98 ± 0.76^{c}	157.89 ± 1.48^{c}	63.12 ± 0.75^{c}
	10%	48.95 ± 2.077	42.89 ± 4.67	5.29 ± 0.05^{b}	39.02 ± 0.29^{b}	67.15 ± 0.77^{b}	139.73 ± 1.49^{b}	57.86 ± 0.76^{b}
Incubation 2 h								
BULLXcell®		36.70 ± 1.91	38.98 ± 5.12	5.17 ± 0.05	50.91 ± 0.34^{a}	66.11 ± 0.87^{a}	116.14 ± 1.43^{a}	116.14 ± 1.43^{a}
	6%	32.02 ± 1.91	48.26 ± 4.67	5.07 ± 0.05^{a}	47.24 ± 0.30^{b}	74.62 ± 0.76^{b}	136.20 ± 1.25^{b}	136.20 ± 1.25^{b}
LDL	8%	32.42 ± 1.96	43.73 ± 4.33	5.38 ± 0.05^{b}	48.43 ± 0.34^{b}	83.83 ± 0.86^{c}	153.41 ± 1.42^{c}	153.41 ± 1.43^{c}
	10%	35.10 ± 1.87	39.35 ± 4.67	5.24 ± 0.05	46.80 ± 0.32^{b}	77.30 ± 0.81^{b}	144.66 ± 1.32^{d}	144.66 ± 1.32^{b}

TM = total motility (%), ALH = lateral head displacement, LIN = linearity, VAP = average velocity path, VCL = curvilinear velocity, VSL = straight-line velocity

values are presented as Least Squares Means + SE

$^{A,B,a-d}$values within the same column and time of incubation with different letters mean significant differences ($P < 0.05$)

doi: 10.17221/27/2016-CJAS

Table 2. Effect of low-density lipoprotein addition to soybean lecithin-based extender Bioxcell® on motility and viability of frozen-thawed spermatozoa

		Viability	TM	ALH	LIN	VAP	VCL	VSL
Incubation 0 h								
Bioxcell®		61.36 ± 2.91^{A}	51.67± 4.67	3.95 ± 0.04^{a}	50.26 ± 0.32^{a}	61.23 ± 0.80^{a}	109.07 ± 1.26^{a}	56.29 ± 0.79^{a}
LDL	4%	66.87 ± 3.09	54.01 ± 5.12	4.64 ± 0.05^{b}	51.56 ± 0.38	74.45 ± 0.95^{b}	127.02 ± 1.50^{b}	69.02 ± 0.94^{b}
	6%	67.59 ± 2.99	52.27 ± 4.67	4.68 ± 0.05^{b}	51.82 ± 0.36^{b}	75.41 ± 0.91^{b}	127.25 ± 1.43^{b}	69.14 ± 0.90^{b}
	8%	74.20 ± 2.91^{B}	50.34 ± 4.67	4.37 ± 0.05^{c}	50.53 ± 0.35	70.58 ± 0.88^{c}	120.02 ± 1.39^{c}	64.99 ± 0.87^{c}
Incubation 2 h								
Bioxcel®		46.05 ± 2.34	28.91 ± 4.67	3.37 ± 0.04^{a}	45.58 ± 0.41	45.52 ± 0.74^{a}	85.95 ± 1.10^{a}	40.76 ± 0.75^{a}
LDL	4%	48.83 ± 2.64	44.16 ± 5.12	3.84 ± 0.04^{b}	45.00 ± 0.41	54.47 ± 0.74^{b}	99.94 ± 1.11^{b}	49.29 ± 0.76^{b}
	6%	46.77 ± 2.34	42.04 ± 4.67	3.72 ± 0.04^{b}	44.87 ± 0.36^{a}	50.87 ± 0.65^{c}	97.00 ± 0.98^{c}	45.93 ± 0.70^{c}
	8%	54.60 ± 2.48	45.90 ± 4.67	3.67 ± 0.04^{b}	46.43 ± 0.39^{b}	53.66 ± 0.71^{b}	97.78 ± 1.05^{c}	49.43 ± 0.72^{b}

TM = total motility (%), ALH = lateral head displacement, LIN = linearity, VAP = average velocity path, VCL = curvilinear velocity, VSL = straight-line velocity

values are presented as Least Squares Means + SE

$^{A,B,a-c}$values within the same column and time of incubation with different letters mean significant differences ($P < 0.05$)

Table 3. Effect of low-density lipoprotein addition to soybean lecithin-based extender Andromed® on motility and viability of frozen-thawed spermatozoa

		Viability	TM	ALH	LIN	VAP	VCL	VSL
Incubation 0 h								
Andromed®		56.20 ± 2.85^{A}	52.60 ± 5.72	5.13 ± 0.05	42.10 ± 0.29	72.75 ± 0.77^{a}	146.21 ± 1.50^{a}	64.41 ± 0.81^{a}
LDL	4%	68.76 ± 2.94^{B}	61.14 ± 4.67	5.18 ± 0.04^{a}	41.43 ± 0.26^{a}	69.84 ± 0.69	140.24 ± 1.35^{b}	61.52 ± 0.72^{b}
	6%	60.15 ± 2.94	62.87 ± 4.67	4.96 ± 0.05^{b}	41.81 ± 0.29	68.73 ± 0.76^{b}	140.29 ± 1.48^{b}	60.72 ± 0.79^{b}
	8%	62.31 ± 2.72	54.45 ± 4.05	5.03 ± 0.05	42.68 ± 0.28^{b}	71.32 ± 0.76	132.45 ± 1.46^{c}	70.10 ± 0.79^{c}
Incubation 2 h								
Andromed®		45.67 ± 2.25	40.58 ± 5.72	4.50 ± 0.04^{a}	41.63 ± 0.33	59.76 ± 0.72^{a}	118.13 ± 1.25^{a}	52.32 ± 0.74^{a}
LDL	4%	44.49 ± 2.20	44.27 ± 4.67	4.38 ± 0.04^{a}	41.07 ± 0.32^{a}	56.42 ± 0.71^{b}	112.86 ± 1.23^{b}	49.54 ± 0.73^{b}
	6%	44.23 ± 2.65	53.32 ± 4.67	4.59 ± 0.04^{a}	42.61 ± 0.30	62.18 ± 0.66^{c}	121.55 ± 1.15^{c}	55.21 ± 0.67^{c}
	8%	45.80 ± 2.32	45.43 ± 4.05	4.74 ± 0.04^{b}	42.77 ± 0.31^{b}	63.34 ± 0.69^{c}	123.59 ± 1.18^{c}	56.45 ± 0.70^{c}

TM = total motility (%), ALH = lateral head displacement, LIN = linearity, VAP = average velocity path, VCL = curvilinear velocity, VSL = straight-line velocity

values are presented as Least Squares Means + SE

$^{A,B,a-c}$values within the same column and time of incubation with different letters mean significant differences ($P < 0.05$)

DISCUSSION

Specific difficulties in using complete egg yolk in extenders (Wall and Foote 1999) led researchers to perform experiments to examine the possibility of egg yolk replacement in extenders with LDL. In most studies, substitution of egg yolk with 8% LDL improved the qualitative parameters of frozen-thawed spermatozoa immediately after thawing in comparison to extenders with whole egg yolk (Moussa et al. 2002; Hu et al. 2010, 2011; Stadnik et al. 2015) and soy bean lecithin-based

doi: 10.17221/27/2016-CJAS

extenders as well (Amirat et al. 2005; Vera-Munoz et al. 2009).

In our study, we modified the protocol of LDL preparation by addition of sodium azide for the long-term preservation of LDL. To the best of our knowledge, other authors (e.g. Moussa at al. 2002; Hu et al. 2010) did not use any preservative in their studies. We achieved similar results as the above-mentioned authors, i.e. a comparable TM, proportion of viable spermatozoa, and better kinematic parameters after substitution of egg yolk with LDL preserved with sodium azide. Moreover, 8% LDL improved the kinematic parameters of spermatozoa, which is in agreement with the results of other authors (e.g. Moussa et al. 2002; Hu et al. 2011). Generally, in these studies, spermatozoa parameters were assessed only immediately after thawing. Our results also confirmed the positive effects of LDL after a 2-hour thermoresistance test. The consensus with other published experiments and, moreover, the positive effects after prolonged spermatozoa incubation under stressful conditions point out standard quality of our own produced LDL. In summary, our modification means an extended period of LDL storage is possible, which is advantageous for its practical use. Moreover, sodium azide should minimize any microbial risk (Hyldgaard et al. 2014), which has been a debatable issue (Aires et al. 2003).

The main objective of our study was to determine the influence of LDL addition on the cryoprotective properties of the two soybean lecithin-based extenders. In the case of the first one (Bioxcell®), our results showed a positive effect of 8% LDL on sperm viability immediately after thawing. Regarding spermatozoa motility, 4%, 6%, and 8% LDL had a beneficial effect on the majority of sperm kinematic parameters immediately after thawing and after the 2-hour thermoresistance test. Nevertheless, the second lecithin-based extender (Andromed®) did not show these trends. Although the addition of 4% LDL had a positive effect on spermatozoa viability after thawing, this effect was not seen in other groups or after the thermoresistance test. Furthermore, the sperm motility parameters did not show any obvious positive tendency as in the Bioxcell® extender. It was shown that there could be a different level of specific interactions between LDL and soy bean lecithin. These differences may be caused by different compositions of the extenders tested in our study, when they may probably differ in percentage of soybean lecithin content; unfortunately more detailed information about this is not available. Low-density lipoprotein, as well as soybean lecithin, acts mainly due to its phospholipid content (Thun et al. 2002). As Moussa et al. (2002) noted, there is a possible interference between the concentration and the positive influence of LDL. In their study, more than 10% LDL as the replacement for egg yolk in the extender led to a decrease in the sperm motility after thawing. The situation was similar in our study, in which 10% LDL in the egg yolk extender BULLXcell® decreased the majority of the sperm kinematic parameters in comparison to the optimal 8% concentration of LDL. Moussa et al. (2002) hypothesized that this was due to precipitation of fructose and salts in the extender. Thereby, the positive effects of LDL addition to already present lecithin in the extender might be also limited.

CONCLUSION

Based on the results of this study it could be concluded that produced LDL might be preserved using sodium azide. And especially that the cryoprotective properties of the semen extenders based on a soybean lecithin could be improved by egg yolk LDL addition. Nevertheless, the identification of the limits or patterns of this synergism are beyond the scope of this study. Additional experiments should be performed to determine optimal LDL concentrations for individual types of soybean lecithin-based extenders, as well as to evaluate the clinical significance of the positive effects observed.

Acknowledgement. We would like to express thanks to Natural Ltd. for cooperation in this work.

REFERENCES

Aires V.A., Hinsch K.D., Mueller-Schloesser F., Bogner K., Mueller-Schloesser S., Hinsch E. (2003): In vitro and in vivo comparison of egg yolk-based and soybean lecithin-based extenders for cryopreservation of bovine semen. Theriogenology, 60, 269–279.

Alomar M., Alzoabi M., Zarkawi M. (2016): Kinetics of hydrogen peroxide generated from live and dead ram spermatozoa and the effects of catalase and oxidase substrates addition. Czech Journal of Animal Science, 61, 1–7.

doi: 10.17221/27/2016-CJAS

Amirat L., Anton M., Tainturier D., Chatagnon G., Battut I., Courtens J.L. (2005): Modifications of bull spermatozoa induced by three extenders: Biociphos, low density lipoprotein and Triladyl, before, during and after freezing and thawing. Reproduction, 129, 535–543.

Amirat-Briand L., Bencharif D., Vera-Munoz O., Ali H.B.H., Destrumelle S., Desherces S., Schmidt E., Anton M., Tainturier D. (2009): Effect of glutamine on post-thaw motility of bull spermatozoa after association with LDL (low density lipoproteins) extender: preliminary results. Theriogenology, 71, 1209–1214.

Amirat-Briand L., Bencharif D., Vera-Munoz O., Pineau S., Thorin C., Destrumelle S., Desherces S., Anton M., Jouan M., Shmitt E., Tainturier D. (2010): In vivo fertility of bull semen following cryopreservation with an LDL (low density lipoprotein) extender: preliminary results of artificial inseminations. Animal Reproduction Science, 122, 282–287.

Beran J., Stadnik L., Bezdicek J., Louda F., Citek J., Duchacek J. (2012): Effect of sire and extender on sperm motility and share of live or dead sperm in bull's fresh ejaculate and in AI doses after thawing. Archiv Tierzücht, 3, 207–218.

Beran J., Stadnik L., Duchacek J., Okrouhla M., Dolezalova M., Kadlecova V., Ptacek M. (2013): Relationships among the cervical mucus urea and acetone, accuracy of insemination timing, and sperm survival in Holstein cows. Animal Reproduction Science, 142, 28–34.

Bergeron A., Crete M.H., Brindle Y., Manjunath P. (2004): Low-density lipoprotein fraction from hen's egg yolk decreases the binding of the major proteins of bovine seminal plasma to sperm and prevents lipid efflux from the sperm membrane. Biology of Reproduction, 70, 708–717.

Crespilho A.M., Sa Filho M.F., Dell'Aqua J.A., Nichi Jr. M., Monteiro G.A., Avanzi B.R., Martins A., Papa F.O. (2012): Comparison of in vitro and in vivo fertilizing potential of bovine semen frozen in egg yolk or new lecithin based extenders. Livestock Science, 149, 1–6.

Drobnis E.Z., Crowe L.M., Berger T., Anchordoguy T.J., Overstreet J.W., Crowe J.H. (1993): Cold shock damage is due to lipide phase transitions in cell membranes – a demonstration using sperm as a model. Journal of Experimental Zoology, 265, 432–437.

Dzyuba V., Cosson J., Dzyuba B., Rodina M. (2015): Oxidative stress and motility in tench Tinca tinca spermatozoa. Czech Journal of Animal Science, 60, 250–255.

Gil J., Januskauskas A., Haard M.C., Haard M.G.M., Johanisson A., Soderquist L., Rodriguez-Martinez H. (2000): Functional sperm parameters and fertility of bull semen extended in Biociphos-Plus® and Triladyl®. Reproduction in Domestic Animals, 35, 69–77.

Harrison R.A.P, Vickers S.E. (1990): Use of fluorescent probes to assess membrane integrity in mammalian spermatozoa. Journal of Reproduction and Fertility, 88, 343–352.

Hu J.H., Li Q.W., Zan L.S., Jiang Z.L., An J.H., Wang L.Q., Jia Y.H. (2010): The cryoprotective effect of low-density lipoproteins in extenders on bull spermatozoa following freezing-thawing. Animal Reproduction Science, 117, 11–17.

Hu J.H., Jiang Z.L., Lv R.K., Li Q.W., Zhang S.S., Zan L.S., Li Y.K., Li X. (2011): The advantages of low-density lipoproteins in the cryopreservation of bull semen. Cryobiology, 62, 83–87.

Hyldgaard M., Mygind T., Vad B.S., Stenvang M., Otzen D.E., Meyer R.L. (2014): The antimicrobial mechanism of action of epsilon- poly-L-lysine. Applied and Environmental Microbiology, 80, 7758–7770.

Koshimoto C., Gamliel E., Mazur P. (2000): Effect of osmolality and oxygen tension on the survival of mouse sperm frozen to various temperatures in various concentrations of glycerol and raffinose. Cryobiology, 41, 204–231.

Meamar M., Shahneh A.Z., Zamiri M.J., Zeinoaldini S., Kohram H., Hashemi M.R., Asghari S. (2016): Preservation effects of melatonin on the quality and fertility of native Fars rooster semen during liquid storage. Czech Journal of Animal Science, 61, 42–48.

Moussa M., Martinet V., Trimeche A., Tainturier D., Anton M. (2002): Low density lipoproteins extracted from hen egg yolk by an easy method: cryoprotective effect on frozen-thawed bull semen. Theriogenology, 57, 1695–1706.

Muchlisin Z.A., Nadiah W.N., Nadiya N., Fadli N., Hendri A., Khalil M., Siti-Azizah M.N. (2015): Exploration of natural cryoprotectants for cryopreservation of African catfish, Clarias gariepinus, Burchell 1822 (Pisces: Clariidae) spermatozoa. Czech Journal of Animal Science, 60, 10–15.

Pace M.M., Graham E.F. (1974): Components in egg-yolk which protect bovine spermatozoa during freezing. Journal of Animal Science, 39, 1144–1149.

Sieme H., Oldenhof H., Wolkers W.F. (2015): Sperm membrane behaviour during cooling and cryopreservation. Reproduction in Domestic Animals, 50, 20–26.

Spalekova E., Makarevich A., Kubovicova E., Ostro A., Chrenek P. (2014): Effect of caffeine on functions of cooling-stored ram sperm in vitro. Acta Veterinaria Brno, 83, 19–25.

Stadnik L., Rajmon R., Beran J., Simonik O., Dolezalova M., Sichtar J., Stupka R., Folkova P. (2015): Influence of selected factors on bovine spermatozoa cold shock resistance. Acta Veterinaria Brno, 84, 125–131.

Thun R., Hurtado M., Janett F. (2002): Comparison of Biociphos-Plus® and TRIS-egg yolk extender for cryopreservation of bull semen. Theriogenology, 57, 1087–1094.

doi: 10.17221/27/2016-CJAS

van Wagtendonk-de Leeuw A.M., Haring R.M., Kaal-Lansbergen L., den Daas J.H.G. (2000): Fertility results using bovine semen cryopreserved with extenders based on egg yolk and soy bean extract. Theriogenology, 54, 57–67.

Vera-Munoz O., Amirat-Briand L., Diaz T., Vasquez L., Schmidt E., Desherces S., Anton M., Bencharif D., Tainturier D. (2009): Effect of semen dilution to low-sperm number per dose on motility and functionality of cryopreserved bovine spermatozoa using low-density lipoproteins (LDL) extender: Comparison to Triladyl® and Bioxcell®. Theriogenology, 71, 895–900.

Verstegen J., Iguer-Ouada M., Onclin K. (2002): Computer assisted semen analyzers in andrology research and veterinary practice. Theriogenology, 57, 149–179.

Vishwanath R., Shannon P. (2000): Storage of bovine semen in liquid and frozen state. Animal Reproduction Science, 62, 23–53.

Wall R.J., Foote R.H. (1999): Fertility of bull sperm frozen and stored in clarified egg yolk-Tris-glycerol extender. Journal of Dairy Science, 82, 817–821.

Zhang X.G., Hong J.Y., Yan G.J., Wang Y.F., Li Q.W., Hu J.-H. (2015): Association of heat shock protein 70 with motility of frozen-thawed sperm in bulls. Czech Journal of Animal Science, 60, 155–169.

Received: 2016–03–21
Accepted after corrections: 2016–08–16

Corresponding Author

Ing. Ondřej Šimoník, Czech University of Life Sciences Prague, Faculty of Agrobiology, Food and Natural Resources, Department of Veterinary Sciences, Kamýcká 129, 165 21 Prague 6-Suchdol, Czech Republic
Phone: +420 733 791 207, e-mail: simoniko@af.czu.cz

Reproduction in Domestic Animals

Low Density Lipoprotein - important player in increasing cryoprotective efficiency of soybean lecithin-based bull semen extenders

Journal:	*Reproduction in Domestic Animals*
Manuscript ID	Draft
Manuscript Type:	Original Article
Date Submitted by the Author:	n/a
Complete List of Authors:	Simonik, Ondrej; Faculty of Agrobiology, Food and Natural Resources, Czech University of Life Sciences Prague, Department of Veterinary Sciences Beran, Jan; Faculty of Agriculture, University of South Bohemia in České Budějovice, Department of Animal Science Manaskova-Postlerova, Pavla; Institute of Biotechnology of the Czech Republic, Laboratory of Reproductive Biology; Faculty of Agrobiology, Food and Natural Resources, Czech University of Life Sciences Prague, Department of Veterinary Sciences Šichtař, Jiří; Czech University of Life Sciences Prague, Department of Veterinary Sciences Stadnik, Ludek; Faculty of Agrobiology, Food and Natural Resources, Czech University of Life Sciences Prague, Department of Animal Husbandry Tumova, Lucie; Faculty of Agrobiology, Food and Natural Resources, Czech University of Life Sciences Prague, Department of Veterinary Sciences Dolezalova, Martina; Faculty of Agrobiology, Food and Natural Resources, Czech University of Life Sciences Prague, Department of Animal Husbandry Folkova, Petra; Faculty of Agrobiology, Food and Natural Resources, Czech University of Life Sciences Prague, Department of Veterinary Sciences Rajmon, Radko; Czech University of Life Sciences Prague, Department of Veterinary Sciences
Subject Area:	Cryobiology < General reproduction, cattle < Species:, Andrology < General reproduction, Biotechnology < General reproduction

RDA Manuscript Proof

Low Density Lipoprotein - important player in increasing cryoprotective efficiency of soybean lecithin-based bull semen extenders

Running title: LDL increases properties of lecithin-based semen extenders

Šimoník Ondřej[a,c], Beran Jan[b,d], Maňásková-Postlerová Pavla[a,c*], Šichtař Jiří[a], Stádník Luděk[b], Tůmová Lucie[a], Doležalová Martina[b], Folková Petra[a], Rajmon Radko[a]

[a] Czech University of Life Sciences Prague, Faculty of Agrobiology, Food and Natural Resources, Department of Veterinary Sciences, Kamýcká 129, Praha 6 – Suchdol, 165 00, Czech Republic

[b] Czech University of Life Sciences Prague, Faculty of Agrobiology, Food and Natural Resources, Department of Animal Husbandry, Kamýcká 129, Praha 6 – Suchdol, 165 00, Czech Republic

[c] Institute of Biotechnology of the Czech Academy of Sciences, Laboratory of Reproductive Biology, v.v.i., Biocev, Průmyslová 595, Vestec, 252 50, Czech Republic

[d] Department of Animal Science, Faculty of Agriculture, University of South Bohemia in České Budějovice, Studentská 1668, České Budějovice, 370 05, Czech Republic

[*] Corresponding author: Pavla Postlerová, Institute of Biotechnology, Czech Academy of Sciences, v.v.i., Biocev, Průmyslová 595, 252 50 Vestec, Tel. +420-325873797, e-mail: pavlam@ibt.cas.cz

Abstract

Low Density Lipoprotein (LDL) could be an important substance for the improvement of the still questionable cryoprotective efficiency of soybean lecithin (SL) extenders. The aim of this study is to assess the effect of LDL extracted from hen-egg yolk on the cryoprotective properties of SL extenders.

In total, 35 ejaculates were collected and cryopreserved at an insemination centre. The effect of 6% (v/v) LDL addition to the extenders on spermatozoa after thawing was tested. Extender modification was assessed on the basis of sperm motility, plasma membrane, mitochondrial and acrosomal integrity by CASA, flow cytometry and fluorescent microscopy. Based on kinematic parameters derived from CASA, *k*-means cluster analysis was used to classify individual spermatozoon into specific subpopulations (fast, medium-fast and slow). A subpopulation of fast spermatozoa was increased in the presence of LDL in both extenders AndroMed® and Bioxcell® ($P<0.05$). Moreover, the positive effect of LDL on sperm motility was confirmed by decreasing the percentage of sperm in slow subpopulation ($P<0.05$). The effect of LDL addition on the incidence of spermatozoa with intact plasma membrane was not demonstrated in any case of extender used ($P>0.05$). The percentage of sperm with intact acrosome was improved when LDL was added to Bioxcell® ($P<0.05$). On the other hand, addition of LDL to AndroMed® improved mitochondrial intactness after thawing ($P<0.05$). Our results showed that adding LDL to selected SL extenders considerably ameliorated the functional parameters of spermatozoa after thawing. Thus, our study unveiled another possibility for improving the results of bull sperm cryopreservation.

Keywords: *bull; cryopreservation; low density lipoprotein; spermatozoa; sperm quality; reproduction*

1. Introduction

Artificial insemination (AI) in dairy cattle is primarily done with cryopreserved semen. Currently, it is generally accepted that cryopreservation of bull semen is adequately successful. However standard protocols provide just a 50% recovery rate post-thawed (Layek et al. 2016). Efforts to increase the effectiveness of sperm cryopreservation are highly desirable. One of the main methods to reduce the negative impact of cryopreservation and increase quality of insemination doses (ID) is the modification of semen extender composition (Yeste 2016).

The plasma membrane (PM), acrosomal membranes and mitochondria are those primarily threatened areas during cryopreservation, along with reduction of sperm motility (Tapia et al. 2012; Layek et al. 2016). One of the primary changes during cryopreservation is cholesterol losses from PM (Srivastava et al. 2013) which induces changes in its liquid state (Layek et al. 2016). Thus cholesterol obviously plays a substantial role in the physiological functions of PM and other closely related structures (Horokhovatskyi et al. 2016).

Low Density Lipoprotein (LDL) as the major content of egg yolk (EY) plasma interacts with spermatozoa and is responsible for its cryoprotective properties (Hu et al. 2011). EY extenders are frequently used in the bull AI industry (Stradaioli et al. 2007). However, EY presents certain obstacles and thus there has been considerable interest in using alternative extenders (Amirat et al. 2005). Soybean lecithin (SL) in bull semen extenders is the leading option in current practice (Layek et al. 2016). Nevertheless, there is no clear consensus concerning its cryoprotective efficiency when compared to EY (Aires et al. 2003; Crespilho et al. 2012; Murphy et al. 2017). LDL as cryoprotective compound can be isolated in standardized quality and conserved (Moussa et al. 2002; Simonik et al. 2016). Moreover, due to the suggested similar mechanism of action (Vidal et al. 2013; Bergeron et al. 2004) and our preliminary studies (Simonik et al. 2016; Beran et al. 2016), their synergistic effectiveness

is much expected. Furthermore LDL is used as bionanomolecule for transfer therapeutic compounds (Harisa and Alanazi 2014). Thereby, LDL may serve as a tool for increasing the cryoprotective efficiency of SL extenders.

The objective of this study was to prove the use of LDL extracted from hen EY- as an enhancing factor for SL extenders used for bull spermatozoa.

2. Material and methods

The study was carried out according to to directive 2010/63/EU and guidelines of the Czech legislation (directive 208/2004 Sb.).

2.1. LDL extraction

Low-density lipoprotein was prepared with 97% purity according to the methodology of Moussa et al. (2002) with modification in term of LDL prolonged shelf life. Hen eggs were obtained from the controlled breeding program of BIOPHARM Inc. (Jílové u Prahy, Czech Republic). Compared to the established methodology, slight modifications arose from our previous study (Simonik et al. 2016). Sodium azide (0.1%) was used to preserve the LDL produced and eliminate sanitary risks. Before using the LDL, sodium azide was removed by extensive dialysis against PBS.

2.2. Preparation of the extenders

SL extenders, AndroMed® (Minitübe, Tiefenbach Germany) and Bioxcell® (IMV Technologies, L'Aigle, France), were used. Freshly dialysed LDL was then added in selected concentration 6% (v/v) based on our preliminary results (Simonik et al. 2016) to each of the aforementioned extenders. Extenders without LDL were used as a control.

2.3. Collection and processing of semen

Seven bulls (3 Holstein-Friesian and 4 Czech Fleckvieh) were used. Ejaculates from each bull were collected five times. All animals were stationed and ordinarily used for ID production at the AI centre Natural Ltd., Hradištko pod Medníkem, Czech Republic (49.87 °N, 14.42 °E). All sires were of the same age, frequency of collection and bred under the same management system as relates to handling, stabling and feeding system. Each of 35 ejaculates was submitted to the basic assessment done by trained laboratory technicians from the AI centre. Collected semen was divided into samples in relation to the number of experimental variants and cryopreserved by standard methods. Straws were analysed at least one week after the cryopreservation. Before each evaluation, straws were thawed in a water bath for 30 s at 37° C. All analyses were performed after 10 min of sample pre-incubation after thawing at a temperature of 37 °C.

2.4. Evaluation of sperm motility

Sperm motility was assessed with the Computer Assisted Sperm Analysis (CASA) module in the NIS Elements Ar 4.50. (Laboratory Imaging Ltd. Prague, Czech Republic), using a DMK 23UM021 camera (Imaging Source, Germany) with a frame rate of 60 images per second and a stereo microscope (Nikon Eclipse E600, Tokyo, Japan) with a heated plate, magnification x 100. After thawing, semen doses were diluted with Sp-TALP composed according to Parrish et al. (1988).

The final concentration of spermatozoa in samples was adjusted to 20 x 10^6 spermatozoa/ml. Then, 5 µl of the sample was evaluated in a Makler® counting chamber (Sefi Medical Instruments, Haifa, Israel) with a 10 µm depth in six different fields per sample. On average, 200 trajectories per field were analysed. Selected kinematic parameters were analysed as follows: curvilinear velocity (VCL, µm/s), velocity of average path (VAP, µm/s),

straight line velocity (VSL, μm/s), straightness (STR, %), and amplitude of lateral head displacement (ALH, μm). Percentages of progressively motile sperm (PMOT) were measured by thresholds VAP > 30 μm/s and STR > 70 %.

2.5. Flow cytometric assessment

Measurements were made using BD LSR II instrument (Becton Dickinson, San Jose, USA). The SYBR 14 dye was excited by Argon ion 488 nm laser and the MitoTracker by HeNe 633 nm laser. Before each specific sperm parameter evaluation, positive and negative control samples were prepared to proper setting of flow cytometry analysis. Positive controls for SYBR-14 or MitoTracker staining were prepared by standard procedure of Percoll gradient (45/90). Negative controls were prepared according to the methodology of Petrunkina et al. (2010).

2.5.1. Sperm viability assessment

For sperm viability analysis, Live/Dead Sperm Viability kit (Life Technologies, Carlsbad, CA) was used. After thawing, sperm were diluted with HEPES buffer saline to a concentration 1 x 10^6 spermatozoa/ml and 5 μl of each fluorochrome were added, resulting in a final concentration of 100 nM of SYBR-14 and 12 μM of PI. The suspension was then incubated in the dark for 10 min at 37° C. Live cells were detected as SYBR14 positive and their green signal is detected on FL 1 (530/28 nm).

2.5.2. Assessment of mitochondrial integrity

For the evaluation of mitochondrial function, MitoTracker Red CMXRos (Invitrogen, California, USA) was used. To distinguish sperm from debris and other particles in extenders, carboxyfluorescein diacetate (CFDA) (Sigma Aldrich, St. Luis, USA) was used. Spermatozoa

were diluted with Sp-TALP to a concentration 1 x 10^6 spermatozoa/ml. Following that, 5 µl of MitoTracker working solution and 1 µl of CFDA were added; final concentration 10 nM and 1 nM, respectively. Functional mitochondria were MitoTracker positive, emitted red fluorescence and were detected on FL 3 (670 LP).

2.6. Assessment of acrosomal integrity

For evaluation of acrosomal integrity, *Pissum Sativum* agglutinin conjugated with FITC (PSA-FITC) (Sigma Aldrich, St. Louis, USA) was used according to methodology of Hu et al. (2010). Air-dried smears were washed three times with PBS, fixed in methanol (-20° C) for 10 min and again washed three times with PBS. Areas for staining were marked with hydrophobic PAP pen (Sigma Aldrich St. Louis, USA) and slides were incubated with PSA-FITC (200 mg/ml PBS) in a wet chamber for 30 min at 37° C. Finally, Vectashield/DAPI (Vector Laboratories Ltd., Peterborough, UK) was added and the sample was mounted under a coverslip. Evaluation was performed using a (Nikon Eclipse E600, Tokyo, Japan) epifluorescent microscope at magnification 600x. Sperm with intact acrosome were characterized with bright homogenous fluorescence in whole acrosome. For each sample, two replicates were evaluated, amounting to 400 spermatozoa counted per sample.

2.7. Statistical analysis

For the evaluation of sperm kinematic parameters from CASA, *k*-means cluster analysis was used to classify motile spermatozoa into subpopulations. Euclidean distances algorithm processed variables STR, VAP, VCL, VSL, with 20 iterations were used to define three clusters (sub-populations) of sperm. According to computed means of selected variables, individual spermatozoon was afterwards assigned to one of three specific sperm subpopulations: fast, medium-fast and slow (Table 2). To determine differences in the

distribution of these subpopulations, the $\chi 2$ test was used. Percentages of progressively motile sperm and data from flow cytometric assessment were expressed as the mean ± standard error of the mean (SEM). The analysis of variance model (ANOVA) was used, considering the specific treatment LDL addition, as the main variable. Tukey's test was used for comparison of significant effect. Differences in all cases were considered as statistically significant at $P < 0.05$. Statistical analysis was performed in STATISTICA 12 software (StatSoft, Czech Republic).

3. Results

3.1. Effect of LDL addition to SL extenders on progressive motility and distribution of sperm subpopulations

Sperm motility evaluated as a percentage of progressively motile sperm (PMOT) was not affected by LDL addition in both extenders ($P > 0.05$) (Table 1). Cluster analysis of sperm kinematic parameters (total number of 53,871 spermatozoa) revealed the effect of LDL addition. The addition of 6% to AndroMed® and Bioxcell® extenders, increased the percentage of fast spermatozoa subpopulation (AndroMed® control: 31.24%, AndroMed® + 6% LDL 34.64%, Figure 1, $P < 0.05$; Bioxcell® control: 46.55, Bioxcell® + 6% LDL: 49.75, Figure 2, $P < 0.05$). A significantly reduced percentage of slow sperm was observed only in the case of LDL addition to AndroMed® (AndroMed® control: 28.48%, AndroMed® + 6% LDL: 22.75, Figure 1, $P < 0.05$). Medium-fast sperm subpopulation was affected by LDL addition in both extenders (AndroMed® control 40.28%, AndroMed® + 6% LDL 42.62; Bioxcell® control 33.93%, Bioxcell® 31.54% Figure. 1, $P < 0.05$).

3.2. Effects of LDL addition to SL extenders on sperm viability, acrosomal and mitochondrial integrity

All following results are presented in Table 1. The effect of LDL addition to the extender AndroMed® nor Bioxcell® on the incidence of spermatozoa with intact PM was not demonstrated ($P > 0.05$). The percentage of sperm with intact acrosome was improved when LDL was added to Bioxcell® extender ($P < 0.05$) while in the case of AndroMed® with 6% LDL differences were insignificant. The addition of six percent LDL to AndroMed® extender improved mitochondrial intactness after thawing ($P < 0.05$), while in Bioxcell® extender, the differences were insignificant.

4. Discussion

A long period elapsed from the first real experimental evidence that bull sperm could be cryopreserved. Despite the efforts of many research groups to improve cryopreservation protocols for bovine semen, considerable percentages of spermatozoa were irreversible damaged (Srivastava et al. 2013). In light of the facts that i) there are inconsistent data on the cryoprotective efficacy of soybean lecithin (SL) based extenders as the most accepted alternative for egg yolk (EY) (Stradaioli et al. 2007) ii) LDL is cryoprotective compound of egg yolk (Moussa et al. 2002) iii) main method to improve results of sperm cryopreservation is modification of semen extender composition (Holt 2000), our study is aimed to assess the effect of LDL on SL cryoprotective properties.

Attempts to examine the effect of LDL addition to animal protein-free SL extenders when there is commercially available liposome based Optixcell® have been strongly supported by a very current study done by Murphy et al. (2017). The authors compared the protective properties of Optixcell® with different types of semen extenders. The study found that Optixcell® was not as sufficient as SL and EY extenders in protection of sperm during short term storage when progressive motility was evaluated. Moreover, this predominantly field study revealed that the calving rate was highest when EY extender was used for semen

preservation. This supports doubts concerning SL and EY extenders effectiveness (Aires et al. 2003; Crespilho et al. 2012; Murphy et al. 2017) and clearly, shows compound of animal origin provides better cryoprotectivity. The fact that artificial liposome-based extender did not exhibit such protection during semen chilling showed that other methods to improve the cryoprotective properties of bull semen extenders could be found.

Our preliminary results indicated the effect of LDL on the sperm functional parameters before and after cryopreservation (Beran et al. 2016; Simonik et al. 2016). The present study, based on a larger variety of functional parameters, was assessed in a higher number of replicates providing more precisely the impact of LDL on cryoprotective efficiency of SL extenders. Our results showed that in either case of AndroMed® or Bioxcell® extender, LDL addition had a significantly beneficial effect on the percentage of sperm in subpopulations fast, medium-fast and slow (Figure 1 and 2). LDL was shown to be more suitable for preserving sperm motility post-thawed in Bioxcell® since, after its addition the samples exhibited the highest percentage of spermatozoa in fast subpopulation. Moreover, as has been confirmed by Ferraz et al. (2014) the percentage of spermatozoa in this subpopulation correlates with fertilizing ability of sample. Concerning the insignificant effect of LDL on PMOT (Table 1), it was proven that cluster analysis as a method reflecting the heterogeneity of sperm population in samples (Martinez-Pastor et al. 2011) pointed out more precisely the response of cells to modified conditions during cryopreservation (Sichtar et al. 2017).

During cryopreservation, cholesterol and phospholipids are released from LDL particles and consequently interact with PM and form a protective layer (Amirat et al. 2005). Moreover, cholesterol may interact deeper in the PM structure and finely tune it to make PM more resistant to harsh conditions during cryopreservation (Bergeron et al. 2004), and thus it keeps its physiological state for further important moments; e.g. capacitation (Travis and

Kopf 2002) or the sperm-egg interaction (Jankovicova et al. 2016). Interestingly our results showed no significant positive effect of LDL on sperm PM in either extender (Table 1). This is contrary to the LDL effect before cryopreservation on PM assessed by our previous study (Beran et al. 2016) and other studies e. g. (Hu et al. 2011). Above all, our results are in compliance with the study Amirat et al. (2005) in which the authors provided the closest insight into the LDL function in PM stability using scanning electron microscopy. Moreover, its positive effect on sperm motility supports evidence that LDL may protect function of proteins in PM correlated with sperm motility as e. g. A-kinase anchored protein 4 (AKAP4) (Luconi et al. 2011). When this effect could not be detected by selected method of sperm PM integrity assessment. Nevertheless, manifestations of the LDL effect could be specifically hidden in other sperm compartments among others in the acrosome as tightly related to the PM (Perumal et al. 2016). Our results show the significant positive effect of 6% LDL addition to Bioxcell® on intactness of this compartment (Table 1) that is crucial for the successful binding and fertilization of oocytes (Layek et al. 2016). This is supported by evidence that LDL had a positive indirect influence on maintaining the functional status of PM due to its high binding affinity to BSP proteins (Lusignan et al. 2011), thereby preventing destabilization (Srivastava et al. 2013). Our results differ according to the extender used, which could indicate their distinct composition. Moreover, previous studies (Simonik et al. 2016; Murphy et al. 2017) revealed the distinct effectiveness of LDL across different SL extenders and protection by themselves alone, respectively. The high content of polyunsaturated fatty acids in the PM of sperm increased their predisposition to damage caused by Reactive Oxygen Species (ROS) (Tapia et al. 2012). Moreover, as sperm have a limited capacity of antioxidative system (Gharagozloo and Aitken 2011) the content antioxidants in LDL (Alvarez-Rodriguez et al. 2013) and effect on antioxidant activity (Hu et al. 2011) is highly important. Sperm mitochondria are organelles, which are the most

susceptible to the higher levels of ROS usually achieved during cryopreservation (Ferrusola et al. 2009). Currently up to a half of the motile sperm population is irreversibly damaged and lost during cryopreservation (Layek et al. 2016). However, these losses may be higher, since there is a proportion of sperm affected by sub-lethal damage localized on mitochondria (Ferrusola et al. 2009). The results of our study revealed a significant positive effect of LDL on mitochondria integrity. This fact clearly shows that LDL containing antioxidants α-tocopherol and ubiquinol (Alvarez-Rodriguez et al. 2013) represents important enhancing agent for antioxidant activity of SL extenders. This is in accordance with studies in which LDL was used as a substitution of EY (Perumal et al. 2016). It conserved a better mitochondrial function of sperm after thawing, thereby providing better conditions for sperm and leading to a higher probability of fertilization. However, this significant effect on mitochondrial activity was only shown in the case of LDL additions to AndroMed® extender; see Table 1. Inconsistencies in the effect of LDL addition through extenders might be attributed to the supposed different composition of extenders. Unfortunately, information concerning either the precise content of SL or other components in extenders is unavailable. Even more, as Futino et al. (2010) stated, excessive concentrations of SL are not beneficial for sperm. Due to the fact that molecules of LDL also include phosphatidylcholine (Hevonoja et al. 2000) total concentrations of this compound could reach levels inhibiting the positive effects of LDL.

To sum up, LDL maintains functional parameters such as motility, acrosomal and mitochondrial integrity of bull spermatozoa at a higher level after thawing, and thus represents a relevant ameliorative agent for increasing the cryoprotective efficiency of SL extender. Moreover, our results show that its positive effects depend on the type of SL extender used. Future research should be directed to validation of the LDL effect in SL extender on the fertilizing ability of spermatozoa under *in vivo* conditions.

Acknowledgements

This study was supported by National Agency for Agricultural Research grant NAZV QJ1210109, by Internal Grant Agency of Czech University of Life Sciences CIGA Projects No 20162026, by CAS (RVO: 86652036) and project BIOCEV (CZ.1.05/1.1.00/02.0109) from the ERDF. We also would like to express thanks to Prof. Joaquín Gadea for valuable suggestions and critical comments, to Dr. Jan Svoboda for help during flow cytometry analysis and to staff of insemination centre Natural, Ltd.

Declaration of interest

Authors confirm that there is no conflict of interest.

References

Aires, V. A., Hinsch, K. D., Mueller-Schloesser, F., Bogner K., Mueller-Schloesser S., & Hinsch E. (2003). In vitro and in vivo comparison of egg yolk-based and soybean lecithin-based extenders for cryopreservation of bovine semen. *Theriogenology*, **60**, 269-279. https://dx.doi.org/10.1016/S0093-691X(02)01369-9

Alvarez-Rodriguez, M., Alvarez, M., Anel-Lopez, L., Martinez-Rodriguez, C., Martinez-Pastor, F., Borragan, S., Anel, L., & De Paz, P. (2013).The antioxidant effects of soybean lecithin- or low-density lipoprotein-based extenders for the cryopreservation of brown-bear (Ursus arctos) spermatozoa. *Reproduction Fertility and Development*, **25**, 1185-1193. https://dx.doi.org/10.1071/RD12181

Amirat, L., Anton, M., Tainturier, D., Chatagnon, G., Battut, I., & Courtens, J. L. (2005). Modifications of bull spermatozoa induced by three extenders: Biociphos, low density lipoprotein and Triladyl, before, during and after freezing and thawing. *Reproduction*, **129**, 535-543. https://dx.doi.org/10.1530/rep.1.00011

Beran, J., Simonik, O., Rajmon, R., Stadnik, S., Dolezalova, M., Krejcarkova, A., Duchacek, J., & Sichtar J. (2016). Effect of LDL addition into selected bull sperm diluters on resistance of spermatozoa against cold shock. *Acta Universitatis Agriculturae et silviculturae mendelianae brunensis*, **64**, 395-399. https://doi.org/10.11118/actaun201664020395

Bergeron, A., Crete, M. H., Brindle, Y., & Manjunath, P. (2004). Low-density lipoprotein fraction from hen's egg yolk decreases the binding of the major proteins of bovine seminal plasma to sperm and prevents lipid efflux from the sperm membrane. *Biology of Reproduction*, **70**, 708-717. https://doi.org/10.1095/biolreprod.103.022996

Crespilho, A. M., Sa Filho, M. F., Dell'Aqua, J. A., Jr., Nichi, M., Monteiro, G. A., Avanzi, B. R., Martins A., & Papa F. O. (2012). Comparison of in vitro and in vivo fertilizing potential of bovine semen frozen in egg yolk or new lecithin based extenders. *Livestock Science*, **149**, 1-6. https://doi.org/10.1016/j.livsci.2012.05.011

Ferraz, M., Morato, R., Yeste, M., Arcarons, N., Pena, A. I., Tamargo, C., Hidalgo, C. O., Muino, R., & Mogas T. (2014). Evaluation of sperm subpopulation structure in relation to in vitro sperm-oocyte interaction of frozen-thawed semen from Holstein bulls. *Theriogenology*, **81**, 1067-1072. https://doi.org/10.1016/j.theriogenology.2014.01.033

Ferrusola, C. O., Fernandez, L. G., Morrell, J. M., Sandoval, C. S., Garcia, B. M., Rodriguez-Martinez, H., Tapia, J. A., & Pena, F. J. (2009). Lipid Peroxidation, Assessed with BODIPY-C11, Increases after Cryopreservation of Stallion Spermatozoa, is Stallion-dependent and is Related to Apoptotic-like Changes. *Reproduction in Domestic Animals*, **44**, 88-88. https://doi.org/10.1530/REP-08-0484

Futino, D. O., Mendes, M. C. B., Matos, W. N. L., Mondadori, R. G., & Lucci, C. M. (2010). Glycerol, Methyl-Formamide and Dimethyl-Formamide in Canine Semen Cryopreservation. *Reproduction in Domestic Animals*, **45**, 214-220. https://doi.org/10.1111/j.1439-0531.2008.01208.x

Gharagozloo, P., & Aitken, R. J. (2011). The role of sperm oxidative stress in male infertility and the significance of oral antioxidant therapy. *Human Reproduction*, **26**, 1628-1640. https://doi.org/10.1093/humrep/der132

Harisa, G. I., & Alanazi, F. K. (2014). Low density lipoprotein bionanoparticles: From cholesterol transport to delivery of anti-cancer drugs. *Saudi Pharmaceutical Journal*, **22**, 504-515. https://doi.org/10.1016/j.jsps.2013.12.015

Hevonoja, T., Pentikainen, M. O., Hyvonen, M. T., Kovanen, P. T., & Ala-Korpela M. (2000). Structure of low density lipoprotein (LDL) particles: Basis for understanding molecular changes in modified LDL. *Biochimica Et Biophysica Acta-Molecular and Cell Biology of Lipids*, **1488**, 189-210. https://doi.org/10.1016/S1388-1981(00)00123-2

Holt, W. V. (2000). Basic aspects of frozen storage of semen. *Animal Reproduction Science*, **62**, 3-22.

Holt, W. V., & Van Look, K. J. W. (2004). Concepts in sperm heterogeneity, sperm selection and sperm competition as biological foundations for laboratory tests of semen quality. *Reproduction*, **127**, 527-535. https://doi.org/doi: 10.1530/rep.1.00134

Horokhovatskyi, Y., Sampels, S., Cosson, J., Linhart, O., Rodina, M., Fedorov, P., Blecha, M., & Dzyuba, B. (2016). Lipid composition in common carp (Cyprinus carpio) sperm possessing different cryoresistance. *Cryobiology*, **73**, 282-285. https://doi.org/10.1016/j.cryobiol.2016.08.005

Hu, J. H., Jiang, Z. L., Lu, R. K., Li, Q. W., Zhang, S. S., Zan, L. S., Li, Y. K., & Li, X. (2011). The advantages of low-density lipoproteins in the cryopreservation of bull semen. *Cryobiology*, **62**, 83-87. https://doi.org/10.1016/j.cryobiol.2010.12.007

Hu, J. H., Li, Q. W., Zan, L. S., Jiang, Z. L., An, J. H., Wang, L. Q., & Jia, Y. H. (2010). The cryoprotective effect of low-density lipoproteins in extenders on bull spermatozoa following freezing-thawing. *Animal Reproduction Science*, **117**, 11-17. https://doi.org/10.1016/j.anireprosci.2009.04.001

Jankovicova, J., Frolikova, M., Sebkova, N., Simon, M., Cupperova, P., Lipcseyova, D., Michalkova, K., Horovska, L., Sedlacek, R., Stopka, P., Antalikova, J., & Dvorakova-Hortova, K. (2016). Characterization of tetraspanin protein CD81 in mouse spermatozoa and bovine gametes. *Reproduction*, **152**, 785-793. https://doi.org/10.1530/REP-16-0304

Layek, S. S., Mohanty, T. K., Kumaresan, A., & Parks, J. E. (2016). Cryopreservation of bull semen: Evolution from egg yolk based to soybean based extenders. *Animal Reproduction Science*, **172**, 1-9. https://doi.org/10.1016/j.anireprosci.2016.04.013

Luconi, M., Cantini, G., Baldi, E., & Forti, G. (2011). Role of a-kinase anchoring proteins (AKAPs) in reproduction. *Frontiers in Bioscience-Landmark*, **16**, 1315-1330.

Lusignan, M. F., Bergeron, A., Lafleur, M., & Manjunath, P. (2011). The Major Proteins of Bovine Seminal Plasma Interact with Caseins and Whey Proteins of Milk Extender. *Biology of Reproduction*, **85**, 457-464. https://doi.org/10.1095/biolreprod.110.089961

Martinez-Pastor, F., Jorge Tizado, E., Julian Garde, J., Anel, L., & De Paz, P. (2011). Statistical Series: Opportunities and challenges of sperm motility subpopulation analysis. *Theriogenology*, **75**, 783-795. https://doi.org/10.1016/j.theriogenology.2010.11.034

Moussa, M., Martinet, V., Trimeche, A., Tainturier, D., & Anton M. (2002). Low density lipoproteins extracted from hen egg yolk by an easy method: cryoprotective effect on frozen-

thawed bull semen. *Theriogenology*, **57**, 1695-1706. https://doi.org/10.1016/S0093-691X(02)00682-9

Murphy, E. M., Murphy, C., O'Meara, C., Dunne, G., Eivers, B., Lonergan, P., & Fair S. (2017). A comparison of semen diluents on the in vitro and in vivo fertility of liquid bull semen. *Journal of Dairy Science*, **100**, 1541-1554. https://doi.org/10.3168/jds.2016-11646

Parrish, J. J., Suskoparrish, J., Winer, M. A., & First, N. L. (1988). Capacitation of bovine sperm by heparin. *Biology of Reproduction*, **38**, 1171-1180.

Perumal, P., Srivastava, S. K., Ghosh, S. K., Baruah, K. K., Bag, S., Rajoria, J. S., Kumar, K., Rajkhowa, C., Pande, M., & Srivastava, N. (2016). Effects of low-density lipoproteins as additive on quality parameters and oxidative stress following cryopreservation of mithun (Bos frontalis) spermatozoa. *Reproduction in Domestic Animals*, **51**, 708-716. https://doi.org/10.1111/rda.12735

Petrunkina, A. M., Waberski, D., Bollwein, H., & Sieme, H. (2010). Identifying non-sperm particles during flow cytometric analysis: a simple approach. *Reproduction in Domestic Animals*, **45**, 39-39. https://doi.org/10.1016/j.theriogenology.2009.12.006

Sichtar, J., Nehasilova, A., Simonik O., & Bubenickova, F. (2017). Effect of Two Freezing Extenders on Characteristic of Fresh and Frozen-Thawed Semen in Endangered Old Kladruber Stallions - A Pilot Study. *Czech Journal of Animal Science*, **62**, 227-233. https://doi.org/10.17221/76/2016-CJAS

Simonik, O., Rajmon, R., Stadnik, L., Sichtar J., Beran, J., Duchacek, J., Hodek P., & Trefil, P. (2016). Effect of low-density lipoprotein addition to soybean lecithin-based extenders on bull spermatozoa following freezing-thawing - preliminary results. *Czech Journal of Animal Science*, **61**, 560-567. https://doi.org/10.17221/27/2016-CJAS

Srivastava, N., Jerome, A., Srivastava, S. K., Ghosh, S. K., & Kumar, A. (2013). Bovine seminal PDC-109 protein: An overview of biochemical and functional properties. *Animal Reproduction Science*, **138**, 1-13. . https://doi.org/10.1016/j.anireprosci.2013.02.008

Stadnik, L., Rajmon, R., Beran, J., Simonik, O., Dolezalova, M., Sichtar, J., Stupka, R., & Folkova, P. (2015). Influence of selected factors on bovine spermatozoa cold shock resistance. *Acta Veterinaria Brno*, **84**, 125-131. https://doi.org/10.2754/avb201584020125

Stradaioli, G., Noro, T., Sylla, L., & Monaci, M. (2007). Decrease in glutathione (GSH) content in bovine sperm after cryopreservation: Comparison between two extenders. *Theriogenology*, **67**, 1249-1255. https://doi.org/10.1016/j.theriogenology.2007.01.009

Tapia, J. A., Macias-Garcia, B., Miro-Moran, A., Ortega-Ferrusola, C., Salido, G. M., Pena, F. J., & Aparicio, I. M. (2012). The Membrane of the Mammalian Spermatozoa: Much More Than an Inert Envelope. *Reproduction in Domestic Animals*, **47**, 65-75. https://doi.org/10.1111/j.1439-0531.2012.02046.x

Travis, A. J., & Kopf, G. S. (2002). The role of cholesterol efflux in regulating the fertilization potential of mammalian spermatozoa. *Journal of Clinical Investigation*, **110**, 731-736. https://doi.org/10.1172/JCI200216392

Vidal, A. H., Batista, A. M., Da Silva, E. C. B., Gomes, W. A., Pelinca, M. A., Silva, S. V., & Guerra, M. M. P. (2013). Soybean lecithin-based extender as an alternative for goat sperm cryopreservation. *Small Ruminant Research,* **109**, 47-51. https://doi.org/10.1016/j.smallrumres.2012.07.022

Yeste, M. (2016). Sperm cryopreservation update: Cryodamage, markers, and factors affecting the sperm freezability in pigs. Theriogenology, 85, 47-64. https://doi.org/10.1016/j.theriogenology.2015.09.047

Figure legends

Figure 1. Effect of LDL addition to the AndroMed® extender on sperm distribution in clusters after thawing.

Different superscripts in same cluster represent significant differences ($P < 0.05$).

Figure 2. Effect of LDL in Bioxcell® extender on sperm distribution in clusters after thawing.

Different superscripts in same cluster represent significant differences ($P < 0.05$).

Table 1. Effect of LDL addition to soybean lecithin-based extenders on sperm qualitative parameters after thawing.

Experimental group	PMOT (%)	Viability (%)	Acrosome integrity (%)	Mitochondrial integrity (%)
Andromed - control	47.69	32.08 ± 1.29	57.39 ± 2,00	28.97 ± 0.81 [a]
Andromed + **6% LDL**	48.11	31.53 ± 1.34	59.19 ± 1,85	32.09 ± 1.17 [b]
Bioxcell - control	49.06	27.31 ± 1.46	56.86 ± 1.79 [a]	30.46 ± 1.25
Bioxcell + **6% LDL**	50.15	28.53 ± 1.43	63.29 ± 1.61 [b]	31.56 ± 1.40

PMOT – progressively motile; Numbers with different superscripts within a column significantly differ ($P < 0.05$).

Table 2: Characterization of different sperm subpopulations determined by cluster analysis of kinematic parameters of motile spermatozoa. Data are expressed as mean ± SEM.

Cluster	N (%)	VCL (μm/s)	VSL (μm/s)	VAP (μm/s)	STR (%)	ALH (μm)
slow spermatozoa	12066 (22.40%)	119.60±0.19	65.60±0.21	72.76±0.19	88.95 ± 0.14	4.55 ± 0.02
medium fast spermatozoa	19988 (37.11%)	208.94±0.21	95.99±0.16	104.86±0.12	90.95 ± 0.08	6.32 ± 0.01
fast spermatozoa	21807 (40.49%)	255.40±0.19	129.24±0.15	136.27±0.13	94.52 ± 0.05	7.36 ± 0.1

VCL - curvilinear velocity (μm/s), VSL - straight-line velocity (μm/s), VAP - average velocity path (μm/s), STR - straightness (%), ALH - lateral head displacement (μm)

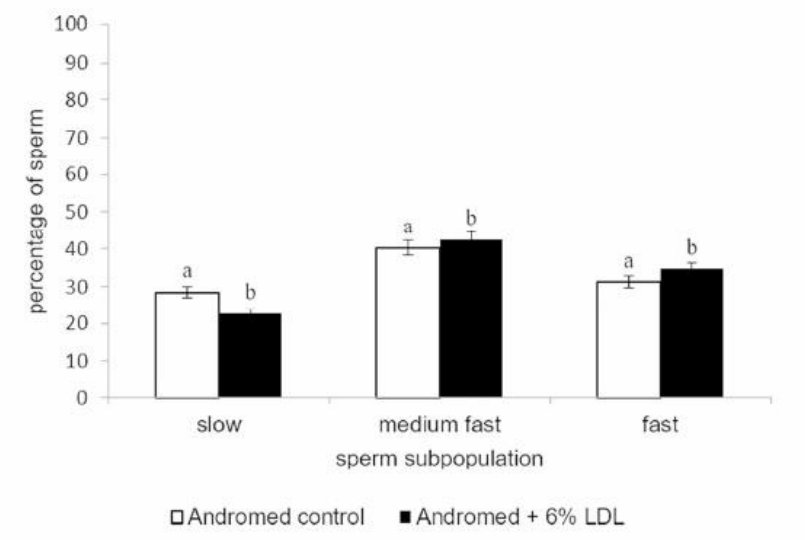


Figure 1

90x61mm (300 x 300 DPI)

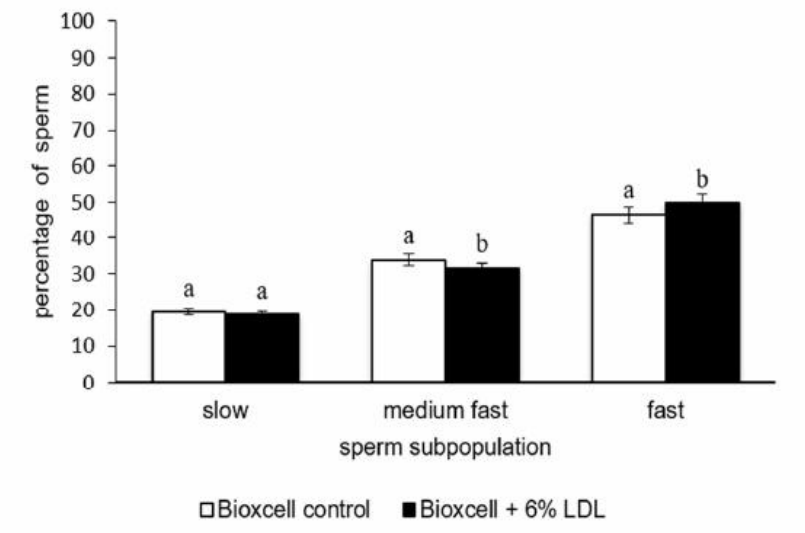


Figure 2

90x61mm (300 x 300 DPI)

5 Sumární diskuze

V současnosti je umělá inseminace kryokonzervovaným semenem nejpoužívanější biotechnologickou metodou v chovu mléčného skotu. Nicméně navzdory dlouhé době, která uplynula od prvních objevů možnosti kryokonzervace spermií býků (Polge et Rowson, 1952), je v současnosti uváděno, že je až padesát procent spermií během tohoto procesu nenávratně poškozeno (Layek et al., 2016). V souvislosti se současným poklesem reprodukční výkonnosti plemenic (Anggraeni et al., 2016) je tedy zcela zřejmé, že snahy o zvýšení efektivity kryokonzervace, a tím výsledně kvality inseminačních dávek jsou velice žádoucí. Jednou z hlavních cest, jak omezit působení negativních vlivů kryokonzervace a zvýšit tak její úspěšnost, je modifikace složení ředidel semene (Holt, 2000a). Prakticky nejpoužívanější alternativou široce využívaných ředidel s obsahem vaječného žloutku (Murphy et al., 2018) jsou ředidla na bázi sójového lecitinu (Muino et al., 2008; Layek et al., 2016). Ta sice eliminují určité problémy, které představuje přítomnost vaječného žloutku (Bousseau et al., 1998; Amirat et al., 2005; Ansari et al., 2010), ovšem studie zabývající se problematikou porovnatelnosti protektivní účinnosti těchto dvou typů ředidel se ve výsledcích rozcházejí např. (Crespilho et al., 2012; Murphy et al., 2017).

Výsledky studie autorů Pace et Graham (1974) jako první ukázaly, že funkční složkou vaječného žloutku odpovědnou za jeho kryoprotektivní vlastnosti je LDL. Po publikaci metodiky pro jeho efektivnější izolaci (Moussa et al., 2002) následovaly další studie, jež na základě substituce vaječného žloutku pomocí LDL v ředidlech semene býků prokázaly jeho pozitivní působení na spermie býků po rozmrazení např. (Hu et al., 2010; Hu et al., 2011). Námi používané LDL bylo produkováno na základě navržené metodiky výzkumným týmem Moussa et al. (2002), která byla ovšem částečně modifikována přidáním azidu sodného, z důvodů možnosti jeho dlouhodobějšího skladování. Součástí naší práce bylo ověření standardních vlastností námi produkovaného LDL. Dle našich výsledků se jako nejoptimálnější, z hlediska motility spermií, pro substituci vaječného žloutku jevila 8% koncentrace LDL, což se shoduje i s pracemi ostatních týmů (Amirat et al., 2004; Hu et al., 2011). Námi připravované LDL tak vykazovalo standardní vlastnosti i po jeho konzervaci, což zvyšuje jeho potenciální praktickou využitelnost.

Naše studie byly primárně zaměřeny na posouzení vlivu adice LDL do ředidel s obsahem sójového lecitinu. Pravděpodobné mechanismy působení LDL a sójového lecitinu jsou obdobné (Bergeron et Manjunath, 2006; Zhang et al., 2009). Ovšem sójový lecitin postrádá ve své

struktuře cholesterol, což je zásadní složka z hlediska rezistence spermií vůči kryokonzervaci (Moce et al., 2010a). Částice LDL kromě vysokého obsahu cholesterolu s sebou nesou také antioxidanty (Anton et al., 2003; Alvarez-Rodriguez et al., 2013). LDL je tak substancí s vysokým potenciálem zvyšovat kryoprotektivní vlastnosti ředidel s obsahem sójového lecitinu.

Negativním faktorům jsou spermie vystaveny již před samotnou kryokonzervací v průběhu postupného zchlazování a ekvilibrace naředěného semene, neboť už v této fázi působí faktory chladového šoku narušující fyziologickou funkci PM (Morris et al., 2012). Vzhledem k tomuto faktu se naše prvotní studie zabývaly vlivem LDL na spermie již v těchto fázích kryokonzervace. Hodnocení odolnosti probíhalo na základě procentuálního zastoupení spermií s intaktní PM, což je primárně poškozovaná struktura v průběhu procesu kryokonzervace (Moce et al., 2010b; Tapia et al., 2012; Khalil et al., 2018). Výsledky prokázaly pozitivní vliv LDL ve spojení s vybranými ředidly na bázi sójového lecitinu Andromed a Bioxcell na rezistenci spermií vůči chladovému šoku. Zjištění pozitivního efektu LDL již v prvotních fázích procesu kryokonzervace nám poskytlo relevantní podklad pro následné testování vlivu LDL ve specifických typech ředidel po vlastní kryokonzervaci.

V průběhu samotné kryokonzervace jsou buňky nadále vystavovány značné zátěži. Snižující se teplota dále vede k přechodu fluidního stavu membrány v krystalickou formu (Sieme et al., 2015) a současně jsou spermie vystavovány fluktuacím osmotického tlaku zapříčiněného tvorbou extracelulárních krystalů (Holt, 2000a; Morris et Acton, 2013). Výsledky předchozích studií ostatních autorů, které byly všechny zaměřeny na možnost náhrady vaječného žloutku v ředidlech ejakulátu býků pomocí LDL, prokázaly jeho pozitivní vliv na řadu funkčních parametrů spermií býků po rozmrazení (Hu et al., 2011; Perumal et al., 2016). Naše prvotní výsledky ukázaly, že adice 6% LDL pozitivně ovlivňovala průměrné hodnoty kinematických parametrů motility spermií, především ve spojení s ředidlem Bioxcell. Co se týče vlivu LDL na viabilitu spermií, nebyla prokázána statistická významnost.

V navazující studii bylo semeno odebráno podstatně většímu počtu býků při několikanásobném opakování a současně byl efekt adice LDL na kvalitu spermií po rozmrazení hodnocen na základě širší škály funkčních parametrů. Tím byla podpořena váha výsledků z hlediska potenciální fertilizační schopnosti spermií (Sellem et al., 2015). Navíc byla analýza vybraných parametrů spermií objektivizována jak použitím průtokové cytometrie (Hossain et al., 2011), tak využitím clusterové analýzy pro vyhodnocení dat z CASA. Tato multiparametrická statistická metoda umožňuje hodnocení vzorku na základě přítomnosti

různých subpopulací spermií (Holt et al., 2007), přičemž její použití bylo iniciováno na základě zpracovaného review (Simonik et al., 2015) a praktických zkušeností ze zahraničních stáží. Clusterová analýza je v současnosti vědeckou společností přijímána jako nejvhodnější statistický postup pro hodnocení kinematických parametrů motility spermií. Zohledňuje heterogenitu populace spermií ve vzorku, což udává její vysokou vypovídající biologickou hodnotu (Holt et Van Look, 2004), a lze tak s její pomocí přesněji odhalit vliv modifikace podmínek při kryokonzervaci (Muino et al., 2008; Sichtar et al., 2017).

Získané výsledky ukázaly signifikantní efekt adice LDL na zastoupení spermií v subpopulacích zahrnující rychlé a pomalé u obou vybraných ředidel. Navíc se LDL jeví jako optimálnější po přidání do ředidla Bioxcell, protože v tomto případě byl nárůst procenta v subpopulaci zahrnující spermie s rychlým pohybem nejvýraznější. Významnost procentuálního zastoupení spermií v této subpopulaci dokládají výsledky studie (Ferraz et al., 2014), pozitivně korelující s výsledky *in vitro* oplození. Co se týče výsledků procenta progresivně motilních spermií (PMOT), nebyl účinek LDL prokázán. To zřetelně ukazuje na velmi obecnou vypovídající schopnost tohoto nejčastěji v praxi hodnoceného parametru spermií.

LDL při nízkých teplotách dosahovaných v průběhu kryokonzervace hraje klíčovou roli při tzv. gelaci vaječného žloutku (Wakamatu et al., 1982; Primacella et al., 2018). Při tomto procesu dochází k rozvolňování struktury LDL a následnému uvolnění obsažených fosfolipidů a cholesterolu LDL do média obklopující spermie, čímž je umožněna jejich interakce s PM a tvorba ochranného filmu (Martinet et al., 2003). Byla prokázána vysoká afinita LDL k vazbě do vnějšího listu PM (Le Guillou et al., 2016), čímž je pozměňován poměr zastoupení cholesterolu k fosfolipidům a tím je docíleno potenciálně vyšší rezistence této struktury vůči kryokonzervaci (Bergeron et al., 2004). Tento princip hraje klíčovou roli v kryoprotektivním působení vaječného žloutku v ředidlech semene (Oldenhof et al., 2015). Vyšší koncentrace cholesterolu v PM jsou schopny pozitivně ovlivňovat rovněž stabilitu proteinových struktur (Sultan, 2010) a udržovat tak jejich biologickou aktivitu související s procesy nezbytnými pro úspěšnou fertilizaci, jako je např. kapacitace (Travis et Kopf, 2002) nebo vazba spermie na oocyt (Jankovicova et al., 2016). Naše výsledky ovšem neukázaly v žádném z vybraných ředidel signifikantně pozitivní vliv adice LDL na integritu PM. To není sice zcela ve shodě s výsledky předešlých studií hodnotících LDL jako substituent vaječného žloutku, ovšem na druhou stranu jsou tyto výsledky obdobné jako v případě studie autorů (Amirat et al., 2005). Ta díky využití elektronové mikroskopie poskytla vůbec nejdetailnější náhled na fungování LDL v rámci PM, výsledky neukázaly významnou podporu její ultrastrukturální integrity. Navíc působení LDL na funkční stav plazmatické membrány může být za možnostmi detekovatelnosti použité metody pro hodnocení. Jeho vliv

může být pozorovatelný na mnohem jemnější úrovni, například ve spojení s udržením integrity proteinových struktur v PM. Ve spojení s našimi výsledky ukazujícími pozitivní vliv LDL na motilitu se jako pravděpodobný jeví pozitivní vliv LDL na A-kinase anchored protein (AKAP4) úzce související s motilitou spermií (Luconi et al., 2011).

Vliv LDL se může projevit v rámci dalších buněčných struktur úzce souvisejících s PM (Perumal et al., 2016). Proto bylo do studie zařazeno i hodnocení efektu LDL na míru poškození akrozómu spermií po rozmrazení. Výsledky ukázaly pozitivní vliv 6% koncentrace LDL v ředidle Bioxcell® na míru intaktnosti této struktury důležité pro úspěšné navázání spermie a oplození oocytu. Tato koncentrace byla prokázána jako optimální z hlediska rezistence spermií vůči chladovému šoku již před kryokonzervací (Beran et al., 2016). Tento pozitivní efekt by mohl být spojen se schopností LDL nepřímo působit na udržení stability a funkční integrity PM. LDL je charakteristické vysokou afinitou k proteinům rodiny BSP (Bovine Sperm Binder), kdy prodloužená expozice spermií těmto proteinům vede k destabilizaci PM (Lusignan et al., 2011a).

Další významnou vlastností LDL jsou antioxidační účinky, které udává obsah antioxidačních složek α-tokoferolu a ubiquinolu (Alvarez-Rodriguez et al., 2013). Vysoký podíl polynenasycených mastných kyselin v PM spermií zvyšuje významně jejich predispozice k poškození volnými kyslíkovými radikály (Tapia et al., 2012). Spermie mají navíc velice omezenou kapacitu antioxidativního systému (Gharagozloo et Aitken, 2011). Bylo prokázáno, že přítomnost LDL pozitivně ovlivňuje aktivitu enzymů katalázy, superoxid dismutázy a glutathion peroxidázy (Gharagozloo et Aitken, 2011). Celková antioxidativní aktivita LDL byla prokázána na vyšší úrovni i v porovnání s ředidly na bázi sójového lecitinu (Alvarez-Rodriguez et al., 2013). Naše výsledky ukázaly významný vliv vybrané 6% koncentrace LDL na zachování integrity mitochondrií. Byla tak dále prokázána vysoká relevantnost adice LDL nesoucího s sebou antioxidanty do ředidel na bázi sójového lecitinu. Přítomnost LDL v těchto ředidlech tak ovlivňuje pozitivně funkční stav mitochondrií spermií po rozmrazení a potenciálně tím zvyšuje jejich fertilizační schopnost. Mitochondrie spermií jsou nejvíce náchylnými organelami spermií na zvýšené hladiny reaktivních forem kyslíku, kterých je během procesu kryokonzervace dosahováno (Yeste, 2015). Je všeobecně uváděno, že co se týče motility, 50 % spermií po rozmrazení ji ztrácí. Ovšem toto procento může být vyšší právě díky nepatrným změnám právě na mitochondriích, jež během posuzování motility není možné zhodnotit. Tento fakt je velice dobře zřetelný i z výsledků naší poslední studie. Tyto sub-letální alterace vedou jak k navýšení procenta spermií se sníženou oplozovací schopností (Layek et al., 2016), tak k negativnímu ovlivnění zbývající populace spermií kvůli nastartování procesů apoptózy (Roca et al., 2016).

Výsledky působení adice LDL na zachování integrity akrozómu a mitochondrií se v kontextu s vybranými ředidly liší. To poukazuje pravděpodobně na jejich rozdílné složení. Tuto hypotézu podporují i výsledky nedávno provedené rozsáhlé studie autorů Murphy et al. (2017), kde samotná SL ředidla vykazovala odlišné protektivní účinky. Lze to vysvětlit možnou interferencí zvýšené koncentrace sójového lecitinu přítomného v ředidlech a přidávaného LDL. Překročení limitního množství fosfatidylcholinu inhibuje jeho pozitivní účinky (Futino et al., 2010). Informace o přesném obsahu sójového lecitinu ve vybraných komerčně vyráběných ředidlech jsou nicméně nedostupné.

Kryobiologie spermií je progresivním vědním oborem a problematika modifikace složení ředidel semene je velice aktuálním tématem. Nedávno bylo na trh uvedeno ředidlo Optixcell (IMV, Francie) s obsahem uměle připravovaných lipozómů s navázaným cholesterolem. Ovšem tato alternativa se na základě výsledků studie Murphy et al. (2017) nejeví zcela jako optimální. Ředidlo neposkytovalo dostatečnou ochranu při uchovávání již ve zchlazeném stavu, v porovnání jak s ředidly žloutkovými, tak na bázi sójového lecitinu. Navíc výsledky prokázaly stejný trend i při testování *in vivo,* kdy se jako nejoptimálnější ukázalo ředidlo s obsahem vaječného žloutku. Tyto výsledky potvrzuje biofyzikální studie, jejíž výstupy ukázaly na signifikantně nižší afinitu lipozómů k PM spermií v porovnání s LDL.

Pokud jde o další alternativu, jak obohatit PM spermií o cholesterol, je to využití cyklodextrinů, na které může být navázán a transportován. Ovšem prozatím se tato možnost nachází stále v experimentální fázi. Bylo však prokázáno, že tato alternativa může mít za následek prodloužení doby potřebné ke kapacitaci spermií, což představuje významný problém z hlediska načasování AI, a tím praktické využitelnosti této alternativy. Ukázaly se tak limitující vlastnosti uměle připravovaných substancí a ve studii Murphy et al. (2017) byly rovněž potvrzeny rozporuplné výsledky porovnatelnosti protektivních účinků ředidel na bázi sójového lecitinu a vaječného žloutku.

Je tedy zřejmé, že naše studie, jež se zabývaly testováním naprosto nové varianty modifikace ředidel s obsahem sójového lecitinu, představují významný krok k možnosti zvýšit jejich kryoprotektivní vlastnosti.

6 Závěr

Účinnost kryoprotektantů obsažených v ředidlech semene je jedním ze zásadních faktorů rozhodujících o úspěšnosti kryokonzervace spermií. LDL, majoritní složka plazmy vaječného žloutku, je charakteristický jak svými výjimečnými protektivními vlastnostmi, tak i specifickou strukturou předurčující ho k jeho použití jako bio-nanomolekuly.

Výsledky našich studií ukázaly, že LDL v interakci s vybranými ředidly na bázi sójového lecitinu Andromed a Bioxcell působí pozitivně již před vlastní kryokonzervací, v průběhu zchlazování spermií. Po kryokonzervaci se pozitivní vliv adice LDL projevil rovněž, a to ve shodné koncentraci 6 %. Efekt LDL po rozmrazení se projevil v distribuci subpopulací motilních spermií, zvýšením procenta spermií s intaktním akrozómem a neporušenou mitochondriální funkcí.

Výsledky substituce vaječného žloutku ve žloutkových ředidlech samotným LDL byly obdobné, jako v případě prací ostatních autorů. Byla tak ověřena standardní kvalita námi produkovaného LDL a zároveň se tímto potvrdila možnost uchovávání LDL v azidu sodném. Bylo tedy umožněno dlouhodobější skladování většího množství LDL získaného z konkrétní šarže vajec, což je důležité z hlediska udržení standardního složení ředidel.
Tento fakt znamená významné podpoření potenciální translace výsledků našeho výzkumu do praxe.

Výzkum byl veden zcela novým směrem zvyšování kryoprotektivní účinnosti ředidel semene býků s obsahem sójového lecitinu. Lze konstatovat, že LDL představuje substanci s významným potenciálem pro zvyšování kryoprotektivních vlastností tohoto typu ředidel. Naše práce otevřela novou cestu ke zvýšení efektivity kryokonzervace spermií býků a zároveň byl vědní obor kryobiologie spermií obohacen o nové poznatky týkající se synergie kryoprotektivního působení LDL a bezžloutkových receptur ředidel na bázi sójového lecitinu.

V budoucnu by bylo vhodné ověřit efekt LDL v navazující *in vivo* studii, případně hlouběji zkoumat jeho působení na proteinové struktury v plazmatické membráně, klíčové z hlediska fertilizační sc

hopnosti spermií.

7 Seznam použité literatury

Agarwal, A., Saleh, R. A., Bedaiwy, M. A. 2003. Role of reactive oxygen species in the pathophysiology of human reproduction. Fertility and Sterility. 79 (4). 829-843.

Aires, V. A., Hinsch, K. D., Mueller-Schloesser, F., Bogner, K., Mueller-Schloesser, S., Hinsch, E. 2003. In vitro and in vivo comparison of egg yolk-based and soybean lecithin-based extenders for cryopreservation of bovine semen. Theriogenology. 60 (2). 269-279.

Aitken, R. J., Baker, M. A. 2004. Oxidative stress and male reproductive biology. Reproduction Fertility and Development. 16 (5). 581-588.

Aksoy, M., Akman, O., Lehimcioglu, N. C., Erdem, H. 2010. Cholesterol-loaded cyclodextrin enhances osmotic tolerance and inhibits the acrosome reaction in rabbit spermatozoa. Animal Reproduction Science. 120 (1-4). 166-172.

Alvarez-Rodriguez, M., Alvarez, M., Anel-Lopez, L., Martinez-Rodriguez, C., Martinez-Pastor, F., Borragan, S., Anel, L., de Paz, P. 2013. The antioxidant effects of soybean lecithin- or low-density lipoprotein-based extenders for the cryopreservation of brown-bear (Ursus arctos) spermatozoa. Reproduction Fertility and Development. 25 (8). 1185-1193.

Amirat, L., Anton, M., Tainturier, D., Chatagnon, G., Battut, I., Courtens, J. L. 2005. Modifications of bull spermatozoa induced by three extenders: Biociphos, low density lipoprotein and Triladyl, before, during and after freezing and thawing. Reproduction. 129 (4). 535-543.

Amirat-Briand, L., Bencharif, D., Vera-Munoz, O., Ali, H. B. H., Destrumelle, S., Desherces, S., Schmidt, E., Anton, M., Tainturier, D. 2009. Effect of glutamine on post-thaw motility of bull spermatozoa after association with LDL (low density lipoproteins) extender: Preliminary results. Theriogenology. 71 (8). 1209-1214.

Anggraeni, A., Herawati, T., Praharani, L., Utami, D., Argis, A. 2016. Conception Rates of Holstein-Friesian Cows Inseminated Artificially with Reducing Frozen Semen Doses. Media Peternakan. 39 (2). 75-81.

Ansari, M. S., Rakha, B. A., Andrabi, S. M. H., Akhter, S. 2010. Usefulness of powdered and fresh egg yolk for cryopreservation of Zebu bull spermatozoa. Reproductive Biology. 10 (3). 235-240.

Anton, M., Martinet, V., Dalgalarrondo, M., Beaurnal, V., David-Briand, E., Rabesona, H. 2003. Chemical and structural characterisation of low-density lipoproteins purified from hen egg yolk. Food Chemistry. 83 (2). 175-183.

Bailey, J., Morrier, A., Cormier, N. 2003. Semen cryopreservation: Successes and persistent problems in farm species. Canadian Journal of Animal Science. 83 (3). 393-401.

Bailey, J. L. 2010. Factors Regulating Sperm Capacitation. Systems Biology in Reproductive Medicine. 56 (5). 334-348.

Benson, J. D., Woods, E. J., Walters, E. M., Critser, J. K. 2012. The cryobiology of spermatozoa. Theriogenology. 78 (8). 1682-1699.

Beran, J., Šimoník, O., Rajmon, R., Stádník, L., Doležalová, M., Krejcárková, A., Ducháček, J., Šichtař, J. 2016. Effect of LDL addition into selected bull sperm diluters on resistance of spermatozoa against cold shock. Acta universitatis agriculturae et silviculturae mendelianae brunensis. 64 (2). 395-399.

Bergeron, A., Crete, M. H., Brindle, Y., Manjunath, P. 2004. Low-density lipoprotein fraction from hen's egg yolk decreases the binding of the major proteins of bovine seminal plasma to sperm and prevents lipid efflux from the sperm membrane. Biology of Reproduction. 70 (3). 708-717.

Bergeron, A., Manjunath, P. 2006. New insights towards understanding the mechanisms of sperm protection by egg yolk and milk. Molecular Reproduction and Development. 73 (10). 1338-1344.

Castro, L. S., Hamilton, T. R. S., Mendes, C. M., Nichi, M., Barnabe, V. H., Visintin, J. A., Assumpcao, M. 2016. Sperm cryodamage occurs after rapid freezing phase: flow cytometry approach and antioxidant enzymes activity at different stages of cryopreservation. Journal of Animal Science and Biotechnology. 7

Celeghini, E. C. C., de Arruda, R. P., de Andrade, A. F. C., Nascimento, J., Raphael, C. F., Rodrigues, P. H. M. 2008. Effects that bovine sperm cryopreservation using two different extenders has on sperm membranes and chromatin. Animal Reproduction Science. 104 (2-4). 119-131.

Cerolini, S., Maldjian, A., Surai, P., Noble, R. 2000. Viability, susceptibility to peroxidation and fatty acid composition of boar semen during liquid storage. Animal Reproduction Science. 58 (1-2). 99-111.

Contri, A., Valorz, C., Faustini, M., Wegher, L., Carluccio, A. 2010. Effect of semen preparation on casa motility results in cryopreserved bull spermatozoa. Theriogenology. 74 (3). 424-435.

Cormier, N., Bailey, J. L. 2003. A differential mechanism is involved during heparin- and cryopreservation-induced capacitation of bovine spermatozoa. Biology of Reproduction. 69 (1). 177-185.

Del Gallego, R., Sadeghi, S., Blasco, E., Soler, C., Yaniz, J. L., Silvestre, M. A. 2017. Effect of chamber characteristics, loading and analysis time on motility and kinetic variables analysed with the CASA-mot system in goat sperm. Animal Reproduction Science. 177 97-104.

Drobnis, E. Z., Crowe, L. M., Berger, T., Anchordoguy, T. J., Overstreet, J. W., Crowe, J. H. 1993. COLD SHOCK DAMAGE IS DUE TO LIPID PHASE-TRANSITIONS IN CELL-MEMBRANES - A DEMONSTRATION USING SPERM AS A MODEL. Journal of Experimental Zoology. 265 (4). 432-437.

El-Sharawy, M., El-Shamaa, I., Ibrahim, M., El-Seify, E. S. 2012. Using of low density lipoproteins and glutamine to improve frozen buffalo bull semen and fertility. Reproduction in Domestic Animals. 47 444-444.

Faezah, S. S. M., Zuraina, F. M. Y., Farah, J. H. F., Khairul, O., Hilwani, N. I., Iswadi, M. I., Fang, C. N., Zawawi, I., Abas, O. M., Fatimah, S. I. 2014. The effects of magnetic separation on cryopreserved bovine spermatozoa motility, viability and cryo-capacitation status. Zygote. 22 (3). 378-386.

Fahy, G. M. 2010. Cryoprotectant toxicity neutralization. Cryobiology. 60 (3). S45-S53.

Ferraz, M., Morato, R., Yeste, M., Arcarons, N., Pena, A. I., Tamargo, C., Hidalgo, C. O., Muino, R., Mogas, T. 2014. Evaluation of sperm subpopulation structure in relation to in vitro sperm-oocyte interaction of frozen-thawed semen from Holstein bulls. Theriogenology. 81 (8). 1067-1072.

Fuchs, B., Mueller, K., Goeritz, F., Blottner, S., Schiller, J. 2007. Characteristic oxidation products of choline plasmalogens are detectable in cattle and roe deer spermatozoa by MALDI-TOF mass spectrometry. Lipids. 42 (11). 991-998.

Futino, D. O., Mendes, M. C. B., Matos, W. N. L., Mondadori, R. G., Lucci, C. M. 2010. Glycerol, Methyl-Formamide and Dimethyl-Formamide in Canine Semen Cryopreservation. Reproduction in Domestic Animals. 45 (2). 214-220.

Gadea, J., Gumbao, D., Canovas, S., Alberto Garcia-Vazquez, F., Alberto Grullon, L., Carlos Gardon, J. 2008. Supplementation of the dilution medium after thawing with reduced glutathione improves function and the in vitro fertilizing ability of frozen-thawed bull spermatozoa. International Journal of Andrology. 31 (1). 40-49.

Gadea, J., Gumbao, D., Gomez-Gimenez, B., Carlos Gardon, J. 2013. Supplementation of the thawing medium with reduced glutathione improves function of frozen-thawed goat spermatozoa. Reproductive Biology. 13 (1). 24-33.

Gadea, J., Molla, M., Selles, E., Marco, M. A., Garcia-Vazquez, F. A., Gardon, J. C. 2011. Reduced glutathione content in human sperm is decreased after cryopreservation: Effect of the addition of reduced glutathione to the freezing and thawing extenders. Cryobiology. 62 (1). 40-46.

Gadella, B. M., Gadella, T. W. J., Colenbrander, B., Vangolde, L. M. G., Lopescardozo, M. 1994. VISUALIZATION AND QUANTIFICATION OF GLYCOLIPID POLARITY DYNAMICS IN THE PLASMA-MEMBRANE OF THE MAMMALIAN SPERMATOZOON. Journal of Cell Science. 107 2151-2163.

Gadella, B. M., Luna, C. 2014. Cell biology and functional dynamics of the mammalian sperm surface. Theriogenology. 81 (1). 74-84.

Gharagozloo, P., Aitken, R. J. 2011. The role of sperm oxidative stress in male infertility and the significance of oral antioxidant therapy. Human Reproduction. 26 (7). 1628-1640.

Gil, J., Januskauskas, A., Haard, M. C., Haard, M. G. M., Johanisson, A., Soderquist, L., Rodriguez-Martinez, H. 2000. Functional sperm parameters and fertility of bull semen extended in biociphos-Plus (R) and Triladyl (R). Reproduction in Domestic Animals. 35 (2). 69-77.

Gorgas, K., Teigler, A., Komljenovic, D., Just, W. W. 2006. The ether lipid-deficient mouse: Tracking down plasmalogen functions. Biochimica Et Biophysica Acta-Molecular Cell Research. 1763 (12). 1511-1526.

Graham, J. K., Kunze, E., Hammerstedt, R. H. 1990. ANALYSIS OF SPERM CELL VIABILITY, ACROSOMAL INTEGRITY, AND MITOCHONDRIAL-FUNCTION USING FLOW-CYTOMETRY. Biology of Reproduction. 43 (1). 55-64.

Griveau, J. F., LeLannou, D. 1997. Reactive oxygen species and human spermatozoa: physiology and pathology. International Journal of Andrology. 20 (2). 61-69.

Hammerstedt, R. H., Graham, J. K., Nolan, J. P. 1990. CRYOPRESERVATION OF MAMMALIAN SPERM - WHAT WE ASK THEM TO SURVIVE. Journal of Andrology. 11 (1). 73-88.

Harisa, G. I., Alanazi, F. K. 2014. Low density lipoprotein bionanoparticles: From cholesterol transport to delivery of anti-cancer drugs. Saudi Pharmaceutical Journal. 22 (6). 504-515.

Hevonoja, T., Pentikainen, M. O., Hyvonen, M. T., Kovanen, P. T., Ala-Korpela, M. 2000. Structure of low density lipoprotein (LDL) particles: Basis for understanding molecular changes in modified LDL. Biochimica Et Biophysica Acta-Molecular and Cell Biology of Lipids. 1488 (3). 189-210.

Holt, W. V. 2000. Fundamental aspects of sperm cryobiology: The importance of species and individual differences. Theriogenology. 53 (1). 47-58.

Holt, W. V., O'Brien, J., Abaigar, T. 2007. Applications and interpretation of computer-assisted sperm analyses and sperm sorting methods in assisted breeding and comparative research. Reproduction Fertility and Development. 19 (6). 709-718.

Holt, W. V., Van Look, K. J. W. 2004. Concepts in sperm heterogeneity, sperm selection and sperm competition as biological foundations for laboratory tests of semen quality. Reproduction. 127 (5). 527-535.

Hossain, M. S., Johannisson, A., Wallgren, M., Nagy, S., Siqueira, A. P., Rodriguez-Martinez, H. 2011. Flow cytometry for the assessment of animal sperm integrity and functionality: state of the art. Asian Journal of Andrology. 13 (3). 406-419.

Hu, J. H., Li, Q. W., Zan, L. S., Jiang, Z. L., An, J. H., Wang, L. Q., Jia, Y. H. 2010. The cryoprotective effect of low-density lipoproteins in extenders on bull spermatozoa following freezing-thawing. Animal Reproduction Science. 117 (1-2). 11-17.

Hyldgaard, M., Mygind, T., Vad, B. S., Stenvang, M., Otzen, D. E., Meyer, R. L. 2014. The Antimicrobial Mechanism of Action of Epsilon- Poly-L-Lysine. Applied and Environmental Microbiology. 80 (24). 7758-7770.

Chatterjee, S., de Lamirande, E., Gagnon, C. 2001. Cryopreservation alters membrane sulfhydryl status of bull spermatozoa: Protection by oxidized glutathione. Molecular Reproduction and Development. 60 (4). 498-506.

Chen, B. B., Li, S. S., Yan, Y. P., Duan, Y. C., Chang, S. H., Wang, H., Ji, W. Z., Wu, X., Si, W. 2017. Cryopreservation of cynomolgus macaque (Macaca fascicularis) sperm with glycerol and ethylene glycol, and its effect on sperm-specific ion channels - CatSper and Hv1. Theriogenology. 104 37-42.

James, P. S., Hennessy, C., Berge, T., Jones, R. 2004. Compartmentalisation of the sperm plasma membrane: a FRAP, FLIP and SPFI analysis of putative diffusion barriers on the sperm head. Journal of Cell Science. 117 (26). 6485-6495.

Jankovicova, J., Frolikova, M., Sebkova, N., Simon, M., Cupperova, P., Lipcseyova, D., Michalkova, K., Horovska, L., Sedlacek, R., Stopka, P., Antalikova, J., Dvorakova-Hortova, K. 2016. Characterization of tetraspanin protein CD81 in mouse spermatozoa and bovine gametes. Reproduction. 152 (6). 785-793.

Khalil, W. A., El-Harairy, M. A., Zeidan, A. E. B, Hassan, M. A. E. 2018. Evaluation of bull spermatozoa during and after cryopreservation: Structural and ultrastructural insights. International Journal of Veterinary Science and Medicine. (in press)

Koshimoto, C., Gamliel, E., Mazur, P. 2000. Effect of osmolality and oxygen tension on the survival of mouse sperm frozen to various temperatures in various concentrations of glycerol and raffinose. Cryobiology. 41 (3). 204-231.

Layek, S. S., Mohanty, T. K., Kumaresan, A., Parks, J. E. 2016. Cryopreservation of bull semen: Evolution from egg yolk based to soybean based extenders. Animal Reproduction Science. 172 1-9.

Le Guillou, J., Ropers, M. H., Gaillard, C., David-Briand, E., Desherces, S., Schmitt, E., Bencharif, D., Amirat-Briand, L., Tainturier, D., Anton, M. 2013. Organization of lipids in the artificial outer membrane of bull spermatozoa reconstructed at the air-water interface. Colloids and Surfaces B-Biointerfaces. 108 246-254.

Le Guillou, J., Ropers, M. H., Gaillard, C., David-Briand, E., van Leeuwen-Ibarrola, J., Desherces, S., Schmitt, E., Bencharif, D., Amirat-Briand, L., Anton, M., Tainturier, D. 2016. Sequestration of bovine seminal plasma proteins by different assemblies of phosphatidylcholine: A new technical approach. Colloids and Surfaces B-Biointerfaces. 140 523-530.

Leite, T. G., do Vale Filho, V. R., de Arruda, R. P., Cesar de Andrade, A. F., Emerick, L. L., Zaffalon, F. G., Matias Martins, J. A., de Andrade, V. J. 2010. Effects of extender and equilibration time on post-thaw motility and membrane integrity of cryopreserved Gyr bull semen evaluated by CASA and flow cytometry. Animal Reproduction Science. 120 (1-4). 31-38.

Lenzi, A., Gandini, L., Picardo, M., Tramer, F., Sandri, G., Panfili, E. 2000. Lipoperoxidation damage of spermatozoa polyunsaturated fatty acids (PUFA): Scavenger mechanisms and possible scavenger therapies. Frontiers in Bioscience. 5 E1-E15.

Luconi, M., Cantini, G., Baldi, E., Forti, G. 2011. Role of a-kinase anchoring proteins (AKAPs) in reproduction. Frontiers in Bioscience-Landmark. 16 1315-1330.

Lusignan, M. F., Bergeron, A., Lafleur, M., Manjunath, P. 2011. The Major Proteins of Bovine Seminal Plasma Interact with Caseins and Whey Proteins of Milk Extender. Biology of Reproduction. 85 (3). 457-464.

Macias Garcia, B., Gonzalez Fernandez, L., Ortega Ferrusola, C., Salazar-Sandoval, C., Morillo Rodriguez, A., Rodriguez Martinez, H., Tapia, J. A., Morcuende, D., Pena, F. J. 2011. Membrane Lipids of the Stallion Spermatozoon in Relation to Sperm Quality and Susceptibility to Lipid Peroxidation. Reproduction in Domestic Animals. 46 (1). 141-148.

Malo, C., Gil, L., Gonzalez, N., Cano, R., de Blas, I., Espinosa, E. 2010. Comparing sugar type supplementation for cryopreservation of boar semen in egg yolk based extender. Cryobiology. 61 (1). 17-21.

Manjunath, P., Lefebvre, J., Jois, P. S., Fan, J. J., Wright, M. W. 2009. New Nomenclature for Mammalian BSP Genes. Biology of Reproduction. 80 (3). 394-397.

Martinet, V., Saulnier, P., Beaumal, V., Courthaudon, J. L., Anton, M. 2003. Surface properties of hen egg yolk low-density lipoproteins spread at the air-water interface. Colloids and Surfaces B-Biointerfaces. 31 (1-4). 185-194.

Moce, E., Blanch, E., Tomas, C., Graham, J. K. 2010. Use of Cholesterol in Sperm Cryopreservation: Present Moment and Perspectives to Future. Reproduction in Domestic Animals. 45 57-66.

Moce, E., Purdy, P. H., Graham, J. K. 2010. Treating ram sperm with cholesterol-loaded cyclodextrins improves cryosurvival. Animal Reproduction Science. 118 (2-4). 236-247.

Moore, S. G., Hasler, J. F. 2017. A 100-Year Review: Reproductive technologies in dairy science. Journal of Dairy Science. 100 (12). 10314-10331.

Morris, G. J., Acton, E. 2013. Controlled ice nucleation in cryopreservation - A review. Cryobiology. 66 (2). 85-92.

Morris, G. J., Acton, E., Avery, S. 1999. A novel approach to sperm cryopreservation. Human Reproduction. 14 (4). 1013-1021.

Morris, G. J., Acton, E., Murray, B. J., Fonseca, F. 2012. Freezing injury: The special case of the sperm cell. Cryobiology. 64 (2). 71-80.

Morris, G. J., Faszer, K., Green, J. E., Draper, D., Grout, B. W. W., Fonseca, F. 2007. Rapidly cooled horse spermatozoa: Loss of viability is due to osmotic imbalance during thawing, not intracellular ice formation. Theriogenology. 68 (5). 804-812.

Mortimer, S. T. 2000. CASA - Practical aspects. Journal of Andrology. 21 (4). 515-524.

Moussa, M., Martinet, V., Trimeche, A., Tainturier, D., Anton, M. 2002. Low density lipoproteins extracted from hen egg yolk by an easy method: cryoprotective effect on frozen-thawed bull semen. Theriogenology. 57 (6). 1695-1706.

Murphy, E. M., Murphy, C., O'Meara, C., Dunne, G., Eivers, B., Lonergan, P., Fair, S. 2017. A comparison of semen diluents on the in vitro and in vivo fertility of liquid bull semen. Journal of Dairy Science. 100 (2). 1541-1554.

Murphy, E. M., O'Meara, C., Eivers, B., Lonergan, P., Fair, S. 2018. Comparison of plant- and egg yolk-based semen diluents on *in vitro* sperm kinematics and *in vivo* fertility of frozen thawed bull semen. Animal Reproduction Science. (191) 3. 70 – 75.

O'Flaherty, C., Matsushita-Fournier, D. 2017. Reactive oxygen species and protein modifications in spermatozoa. Biology of Reproduction. 97 (4). 577-585.

Oldenhof, H., Heutelbeck, A., Blasse, A. K., Bollwein, H., Martinsson, G., Wolkers, W. F., Sieme, H. 2015. Tolerance of spermatozoa to hypotonic stress: role of membrane fluidity and correlation with cryosurvival. Reproduction Fertility and Development. 27 (2). 285-293.

Oresti, G. M., Luquez, J. M., Furland, N. E., Aveldano, M. I. 2011. Uneven Distribution of Ceramides, Sphingomyelins and Glycerophospholipids Between Heads and Tails of Rat Spermatozoa. Lipids. 46 (12). 1081-1090.

Pace, M. M., Graham, E. F. 1974. COMPONENTS IN EGG-YOLK WHICH PROTECT BOVINE SPERMATOZOA DURING FREEZING. Journal of Animal Science. 39 (6). 1144-1149.

Parks, J. E., Graham, J. K. 1992. EFFECTS OF CRYOPRESERVATION PROCEDURES ON SPERM MEMBRANES. Theriogenology. 38 (2). 209-222.

Pena, F. J., Macias Garcia, B., Samper, J. C., Aparicio, I. M., Tapia, J. A., Ortega Ferrusola, C. 2011. Dissecting the molecular damage to stallion spermatozoa: The way to improve current cryopreservation protocols? Theriogenology. 76 (7). 1177-1186.

Perumal, P., Srivastava, S. K., Ghosh, S. K., Baruah, K. K., Bag, S., Rajoria, J. S., Kumar, K., Rajkhowa, C., Pande, M., Srivastava, N. 2016. Effects of low-density lipoproteins as additive on quality parameters and oxidative stress following cryopreservation of mithun (Bos frontalis) spermatozoa. Reproduction in Domestic Animals. 51 (5). 708-716.

Petrunkina, A. M., Waberski, D., Bollwein, H., Sieme, H. 2010. Identifying non-sperm particles during flow cytometric physiological assessment: a simple approach. Theriogenology. 73 (7). 995-1000.

Polge, C., Rowson, L. E. A. 1952. FERTILIZING CAPACITY OF BULL SPERMATOZOA AFTER FREEZING AT-79-DEGREES-C. Nature. 169 (4302). 626-627.

Pradeep, K., Srivastava, N., Pande, M., Prasad, J. K., Sirohi, A. S. 2017. In: Srivastava, N., Pande, M. (eds.). Protocols in Semen Biology (Comparing Assays). Springer Nature. Singapore. 57 – 73. ISBN: 978-981-10-5199-9.

Primacella, M., Wang, T., Acevedo, N. C. 2018. Use of Reconstitued Yolk Systems To Study the Gelation Mechanism of Frozen-Thawed Hen Egg Yolk. Journal of Agricultural and Food Chemistry. 66 (2). 512-520.

Purdy, P. H., Graham, J. K. 2004. Effect of cholesterol-loaded cyclodextrin on the cryosurvival of bull sperm. Cryobiology. 48 (1). 36-45.

Roca, J., Parrilla, I., Gil, M. A., Cuello, C., Martinez, E. A., Rodriguez-Martinez, H. 2016. Non-viable sperm in the ejaculate: Lethal escorts for Contemporary viable sperm. Animal Reproduction Science. 169 24-31.

Saez, F., Ouvrier, A., Drevet, J. R. 2011. Epididymis cholesterol homeostasis and sperm fertilizing ability. Asian Journal of Andrology. 13 (1). 11-17.

Salmon, V. M., Leclerc, P., Bailey, J. L. 2016. Cholesterol-Loaded Cyclodextrin Increases the Cholesterol Content of Goat Sperm to Improve Cold and Osmotic Resistance and Maintain Sperm Function after Cryopreservation. Biology of Reproduction. 94 (4).

Sellem, E., Broekhuijse, M. L. W. J., Chevrier, L., Camugli, S., Schmitt, E., Schibler, L., Koenen, E. P. C. 2015. Use of combinations of in vitro quality assessments to predict fertility of bovine semen. Theriogenology. 84 (9). 1447-1454.

Schiller, J., Muller, K., Suss, R., Arnhold, J., Gey, C., Herrmann, A., Lessig, J., Arnold, K., Muller, P. 2003. Analysis of the lipid composition of bull spermatozoa by MALDI-TOF mass spectrometry - a cautionary note. Chemistry and Physics of Lipids. 126 (1). 85-94.

Sieme, H., Oldenhof, H., Wolkers, W. F. 2015. Sperm Membrane Behaviour during Cooling and Cryopreservation. Reproduction in Domestic Animals. 50 20-26.

Signorelli, J., Diaz, E. S., Morales, P. 2012. Kinases, phosphatases and proteases during sperm capacitation. Cell and Tissue Research. 349 (3). 765-782.

Sichtar, J., Nehasilova, A., Simonik, O., Bubenickova, F. 2017. Effect of Two Freezing Extenders on Characteristic of Fresh and Frozen-Thawed Semen in Endangered Old Kladruber Stallions - A Pilot Study. Czech Journal of Animal Science. 62 (6). 227-233.

Simonik, O., Rajmon, R., Stadnik, L., Sichtar, J., Beran, J., Duchacek, J., Hodek, P., Trefil, P. 2016. Effect of low-density lipoprotein addition to soybean lecithin-based extenders on bull spermatozoa following freezing-thawing - preliminary results. Czech Journal of Animal Science. 61 (12). 560-567.

Simonik, O., Sichtar, J., Krejcarkova, A., Rajmon, R., Stadnik, L., Beran, J., Dolezalova, M., Biniova, Z. 2015. Computer assisted sperm analysis - the relationship to bull field fertility, possible errors and their impact on outputs: A review. Indian Journal of Animal Sciences. 85 (1). 3-11.

Singh, V. K., Kumar, R., Atreja, S. K. 2014. Cryo-survival, cryo-capacitation and oxidative stress assessment of buffalo spermatozoa cryopreserved in new soya milk extender. Livestock Science. 160 214-218.

Sparr, E., Hallin, L., Markova, N., Wennerstrom, H. 2002. Phospholipid-cholesterol bilayers under osmotic stress. Biophysical Journal. 83 (4). 2015-2025.

Srivastava, N., Jerome, A., Srivastava, S. K., Ghosh, S. K., Kumar, A. 2013. Bovine seminal PDC-109 protein: An overview of biochemical and functional properties. Animal Reproduction Science. 138 (1-2). 1-13.

Srivastava, N., Srivastava, S. K., Ghosh, S. K., Kumar, A., Jerome, A., Singh, M., Ramteke, S. S. 2015. Cryoinjury in relation to cholesterol content of bull spermatozoa. Indian Journal of Animal Research. 49 (3). 308-312.

Srivastava, N., Srivastava, S. K., Ghosh, S. K., Singh, L. P., Prasad, J. K., Kumar, A., Perumal, P., Jerome, A., Thamizharasan, A. 2012. Sequestration of PDC-109 protein improves freezability of crossbred bull spermatozoa. Animal Reproduction Science. 131 (1-2). 54-62.

Sultan, S. D. 2010. Perspective on plasma membrane cholesterol efflux and spermatozoal function. Journal of Human Reproduction Science. 3 (2). 68-75.

Tannert, A., Toepfer-Petersen, E., Herrmann, A., Mueller, K., Mueller, P. 2007. The lipid composition modulates the influence of the bovine seminal plasma protein PDC-109 on membrane stability. Biochemistry. 46 (41). 11621-11629.

Tapia, J. A., Macias-Garcia, B., Miro-Moran, A., Ortega-Ferrusola, C., Salido, G. M., Pena, F. J., Aparicio, I. M. 2012. The Membrane of the Mammalian Spermatozoa: Much More Than an Inert Envelope. Reproduction in Domestic Animals. 47 65-75.

Therien, I., Moreau, R., Manjunath, P. 1999. Bovine seminal plasma phospholipid-binding proteins stimulate phospholipid efflux from epididymal sperm. Biology of Reproduction. 61 (3). 590-598.

Thibier, M., Wagner, H. G. 2002. World statistics for artificial insemination in cattle. Livestock Production Science. 74 (2). 203-212.

Travis, A. J., Kopf, G. S. 2002. The role of cholesterol efflux in regulating the fertilization potential of mammalian spermatozoa. Journal of Clinical Investigation. 110 (6). 731-736.

van Wagtendonk-de Leeuw, A. M., Haring, R. M., Kaal-Lansbergen, L., den Daas, J. H. G. 2000. Fertility results using bovine semen cryopreserved with extenders based on egg yolk and soy bean extract. Theriogenology. 54 (1). 57-67.

Vera-Munoz, O., Amirat-Briand, L., Diaz, T., Vasquez, L., Schmidt, E., Desherces, S., Anton, M., Bencharif, D., Tainturier, D. 2009. Effect of semen dilution to low-sperm number per dose on motility and functionality of cryopreserved bovine spermatozoa using low-density lipoproteins (LDL) extender: Comparison to Triladyl (R) and Bioxcell (R). Theriogenology. 71 (6). 895-900.

Vishwanath, R. 2003. Artificial insemination: the state of the art. Theriogenology. 59 (2). 571-584.

Wakamatu, T., Sato, Y., Saito, Y. 1982. IDENTIFICATION OF THE COMPONENTS RESPONSIBLE FOR THE GELATION OF EGG-YOLK DURING FREEZING. Agricultural and Biological Chemistry. 46 (6). 1495-1503.

Wathes, D. C., Abayasekara, D. R. E., Aitken, R. J. 2007. Polyunsaturated fatty acids in male and female reproduction. Biology of Reproduction. 77 (2). 190-201.

Watson, P. F. 2000. The causes of reduced fertility with cryopreserved semen. Animal Reproduction Science. 60 481-492.

Whiston, R., Finlay, E. K., McCabe, M. S., Cormican, P., Flynn, P., Cromie, A., Hansen, P. J., Lyons, A., Fair, S., Lonergan, P., O' Farrelly, C., Meade, K. G. 2017. A dual targeted beta-defensin and exome sequencing approach to identify, validate and functionally characterise genes associated with bull fertility. Scientific Reports. 7

Woods, E. J., Benson, J. D., Agca, Y., Critser, J. K. 2004. Fundamental cryobiology of reproductive cells and tissues. Cryobiology. 48 (2). 146-156.

Yeste, M. 2015. Recent Advances in Boar Sperm Cryopreservation: State of the Art and Current Perspectives. Reproduction in Domestic Animals. 50 71-79.

Zhang, S. S., Hu, J. H., Li, Q. W., Jiang, Z. L., Zhang, X. Y. 2009. The cryoprotective effects of soybean lecithin on boar spermatozoa quality. African Journal of Biotechnology. 8 (22). 6476-6480.

8 Přílohy

Příloha č. 1

Product: NIS Elements AR
Version: 4.50 with patch 4
Status: Official

Sperm Motility software and protocol

Revision:

Date	Reviser	Changes
Sep 24, 2014	Ondřej Šimoník	Document Created
Oct 16, 2014	Ondřej Pražský	Document Modified
Mar 23, 2016	Ondřej Pražský	Document Modified

Table of contents:

Introduction

Sperm Motility module is a set of functionality built on the top of the modular JOBS architecture.

Its goal is to perform the sperm motility analysis from the acquisition to the final exports.

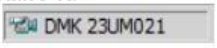
The tools are specifically designed for:

- acquiring different kind of time-lapse experiments,
- associating metadata with different specimen conditions,
- running custom automated or supervised decision guided acquisition schemes,
- managing and inspecting acquired data,
- analyzing acquired data for individual sperm motility features,
- showing motility statistics and graphs,
- classifying based on any measured feature,
- showing the results in graph or
- exporting for further analysis

NIS-Elements check

NOTE: Do this before each experimental day!

1. USB Dongle inserted.
2. Enough free space on the acquisition disk: at least 20GB before experiment
3. Correct camera switched ON and running. Confirm with status bar:

   For example: 

4. Correct Optical Configuration set. Confirm with status bar:

5. Check the Calibration.

In case of failure in one or more of the above check see the troubleshooting section.

Plan Carefully

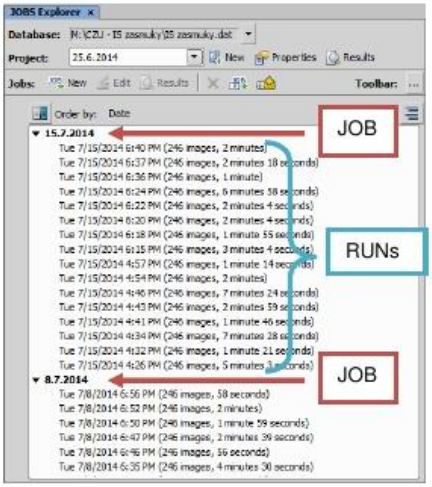
- all relevant sample marking (they can be changed later but there is a big potential for an error)
- the location of the Database (this cannot be changed without having to move the whole database elsewhere)

Preparing a new Database

NOTE: This preparation step can skipped if the acquisition will be done into already created database.

All data are acquired into a Database. It has following structure.

The database must be created and configured first. Here is a sample image of already populated JOBS Explorer window:



A. New database

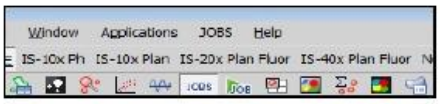
The database is residing in its (database) root folder. It will contain all the image data and the metadata (labels, measurements, tracks etc.) stored into the database (the DAT file) throughout whole experiment.

The inner structure of the folders and files inside the database root folder must be kept unaltered as it may lead to data loss.

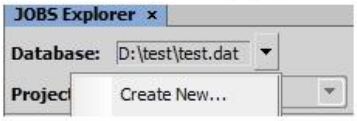
Choose the names and folders carefully!

In NIS-Elements select from menu “JOBS” the second item “Explorer...” It will stay open during whole session.

NOTE: You do most of interactions from within this window.



The new database is created by selecting "Create New..." from the popup menu.



NOTE: The same functionality can be found in the JOBS popup in main menu.

B. Creating a New project

Project is a logical category into which users may group their data if it is helpful for their organizational needs.

Database cane divided into projects which make different experiment days, for instance.

It is a second highest ranking division under the database.

In order to create a new project, use the "New" button. Fill its new name and some additional information about the type and place of the experiment.

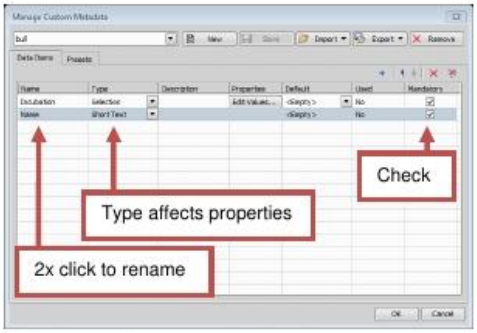
NOTE: Data from different projects cannot be mixed during visualization. They are treated as separate units.

C. Configuring Metadata

Metadata are pieces of information stored together with image data. Depending on what is relevant for the experiment it can be: species, identification markers, incubation time, used chemical etc.

To add new Metadata, use JOBS menu item "Manage Custom Metadata…"

In the dialog choose "New" to make a new Custom Metadata set from scratch or "Import" to load an already premade.



Already made Custom Metadata Sets (here "bull") can be exported into XML files for later use in another database.

D. Creating a New job

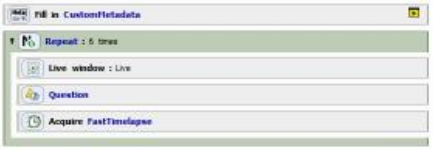
Job is a program that is run in order to acquire data. It produces individual runs.

Job is an organization category too. Each Job can be an experimental day or experimental group. Thus it is created on each such an event.

In order to import a premade job use the "Import" button in the JOBS Explorer.

Another possibility is to create the new job from the scratch.

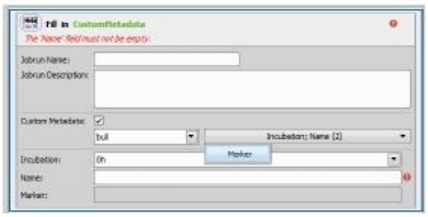
Example: Simple Job for Sperm Motility



The Job (program) consists of five tasks in a special (nested) order:

1. Fill in Custom Metadata (in Runtime)
2. Repeat 6 times the following:
    - Show live image
    - Ask the user to move to different location and wait for ENTER key
    - Run a fast 2second time-lapse

Prefilling the Metadata



While creating the Job, prefill the metadata task carefully. This formulary will be presented to the operartor once more during acquisition, but it is better to solve most of the issuse now:

- Fill Jobrun name and description as you like,
- check Custom Metadata box,
- choose correct group (here "bull"),
- select all fields (and they will cosequently enabled below) and
- fill all mandatory fields (then all exclamation marks will disappear).

Be sure to set it show in runtime (the yellow widow in the top left corner) using the context menu.

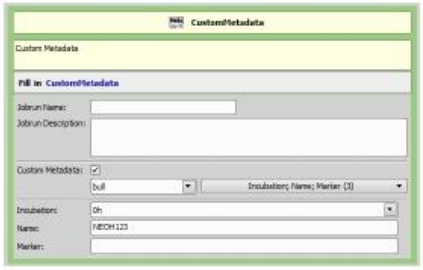
After creating or modificating the job, do not forget to export the Job definition for later use in another database. Use "Save to file…" under the button "Export".

Save your modifications (if needed) and Close the Job definition editor window.

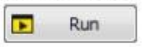
Running jobs

After previous step there is at least one job experiment definition ready to be run in the "JOBS Explorer":

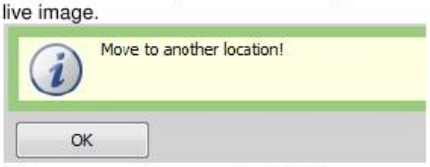
1. Activate a Job by clicking on it.
2. Run the job using the green arrow 
3. Fill the metadata until there are no missing values indicated by red exclamation marks



4. Click the button "Run" to continue the job



5. Move with the manual stage to different location on the sample while checking it on live image.



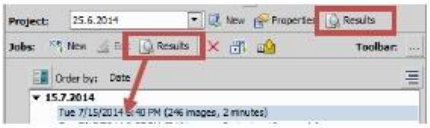
6. This sequence is repeated until all six image fields are acquired.

After every experimental day a backup of acquired should be performed (see backing up).

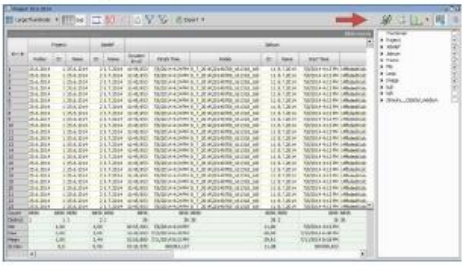
Running analysis

Invocation

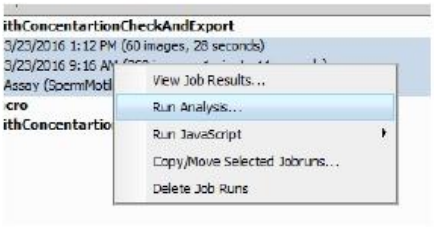
The Sperm Motility analysis is run after the acquisition from the results table (depicted by the arrow).



Ideally the Recipe can be trained for the whole project (all images in a project). If sample variations between experiments are too big, use jobs or job runs.

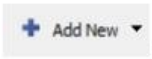


Or on a selection of Jobs or Jobruns from the context menu.



Run analyses window

The analysis window may contain a list of already run analyses ("already run"). They can be inspected, exported or duplicated.



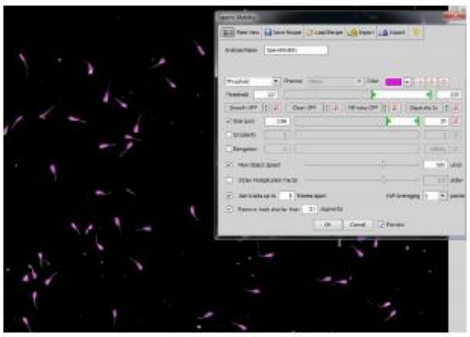
From the popup list select "Sperm Motility" and click the "Define…" or "Redefine…" button.

NOTE: Same analysis name as overwrites the existing analysis. Yellow exclamation icon is displayed.

Or choose a recipe previously saved into the database.

SpermMotility analysis

Open an image file by doubleclicking inside the table and start setting the recipe or load some premade one either from filesystem or from the database.



While doing the next steps observe the changes in overlay binary mask.

NOTE: You can change its color by pressing [Ins] key and trasparency by presing [Ctrl] + [Up] or [Ctrl] + [Dn] keys.

1. Rename the analysis to some meaningful name.

2. Manipulate by the left threshold handle in order to find at which intensity the sperms begin to have a droplet shape.
   Do not include too much tail.
3. Play with Separate (2x or 3x)
4. Set Size, Circularity and Elongation restrictions to proper bounds for given sample.
5. Set the "Maximum Object speed" above maximum frame to frame displacement (usually more than 4x higher than average speed).
6. Use "StDev multiplication factor" to let more sperm to connect from frame to frame.
7. Use "Join tracks up to" to connect tracks that have gaps.
8. Set the "Remove track shorter than" to one plus half of the time-lapse frame count. This way no two tracks will be counted twice.

After having set the parameters scroll inside the time-lapse into different locations to see the quality of detections. It is wise to check like this more than one time-lapse.

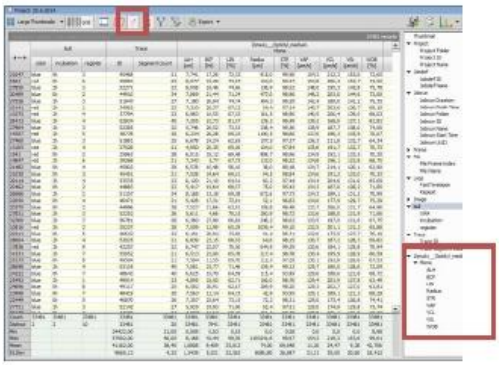
Save the Recipe into the database.

Export the Tracking recipe into a "SpermMotility" recipe file for later use.

3. Save the Tracking Recipe

Close the Recipe definition window and Run it.

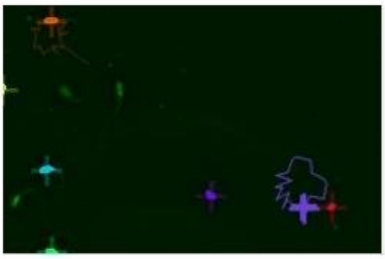
When the Analysis is done a new Table Track view becomes available. Be sure to check the features under the analysis name.



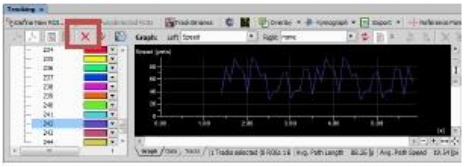
Working with results

Visualization

Results are linked with image data. Any entity (Image, Object or Track) can be inspected by double-clicking a row in the Grid View. The relevant document file is opened on the frame and the object or track is highlighted.



The tracking control is synchronized too.



Individual trajectories may be removed from here.

Statistics

The button adds statistics for each column at the bottom of the table. Ordinal values contain Count and Count of Distinct (or Unique) values. Whereas cardinal values contain also Min, Max, Mean and StDev.

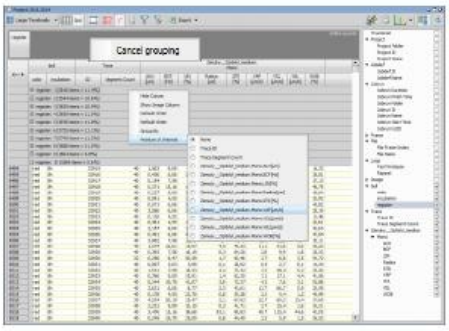
The statistics reflects row selection (when bigger than one row). To signal this mode it turns into selection color.

Sorting

By clicking on the column header, the table will sort based on that column. Second click will sort it in opposite order. The sort is stable, so more columns can be sorted meaningfully.

Grouping

Grouping allows putting together rows with the same value in a given column. Here is an example of grouping according to register.



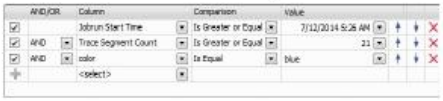
Grouping is made by dragging a column into the dark grey area above the columns. Table can be grouped based on more than one column. To exit from grouping use context menu on the dark gray area again.

Statisticts of a Feature of interest can be displayed for each group.

Filtering

Filtering reduces the amount of records (rows) in the table. Several rules can be added to restrict the rows by column. Individual rules are joined by logical “AND” or “OR" and can be individually switched off.

There are different options for comparisons and values based on the type of the column (e.g. date has a different picker than a real value or an enumeration value).

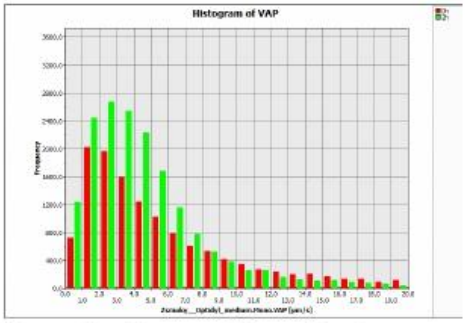


Graphing

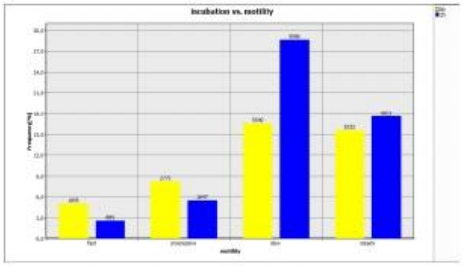
Through the graph button 5 graphs are available: Histogram, Scatterplor, Timechart, Barchart and XY Line Plot.

For sperm motility the histogram, scatterplot and barchart are most useful.

In the next image there is histogram of two populations with different incubation.



The next is a histogram of four motility classes (progressive, fast, slow, steady) vs. two different incubations (0h, 2h).



Each graph type has a slightly different general definition page where it's X and Y axis are defined.

Remaining pages are same for all graphs.

Filter page has the smae function as the filter in the results (see above).

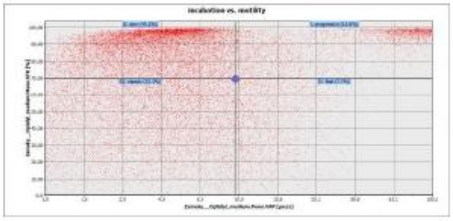
Line series and tiling represent other graph axes. Whereas Line series are diferentiated by colors, tiling is by placing the graphs alongside.

Different options are available for ordinal and cardinal values. While the first can be only selected the others are organized into bins.

Classification

Histogram and Scatterplot allow manual classification (scatterplot also gating).

In this example objects are classified based on the VAP and STR features into four groups (progressive, fast, slow, steady).



Using JavaScript

JavaScrip is a way add functionality to NIS Elements. It involves programming.

From JavaScript it is possible to access the experiment data from the underlying database. Moreover, it is possible to add new data records.

Exapmples

- Classification and statistics displaying the percentage of Motile and Progressive in the sample.
- Customized export for easier statistics.

Invocation

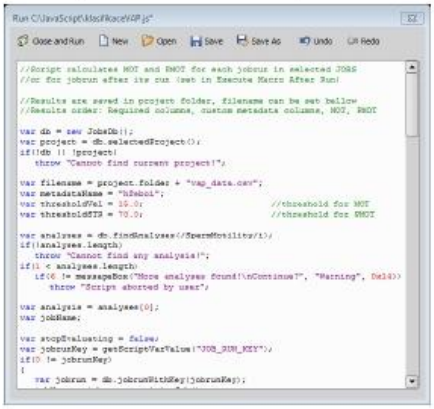
JavaScript can be run or edited from the

- Main JOBS menu, Database menu and Context menu.

- It can be also run from the JOB Task "Execute Macro After Run".

Editor

JavaScript can be edited in a simple editor.



Features

- Query selected project, job or jobrun.

```
var db = new JobsDb();
var project = db.selectedProject();
var selectedJobs = project.selectedJobs();
```

- Query jobrun name, date and metadata.

```
var jobrun = db.jobrunWithKey(jobrunKey);
var name = jobrun.containingJob().name;
var m = jobrun.metadata("Bull");
if (m.incubation == 2 && m.sample == "Neo")
  // ... do something
```

- Query existing analyses and features having been measured.

```
var a = db.findAnalyses(/SpermMotility/i);
var featureSTR =
  analysis.traceFeatureInJobrun(
    jobrun.key, "", "STR");
```

- Create table snapshots, iterate over the rows and columns

```
var table = new TraceTable(
  ["trace.id", featureVel, featureSTR],
  "jobrun.id =" + jobrun.key);

for (row in table)
{
  if (15.0 < table[row][1])
  {
    if (70.0 < table[row][2])
      countPMOT++;
    countMOT++;
  }
  totalCount++;
}

var MOT = countMOT/totalCount*100;
var PMOT = countPMOT/totalCount*100;
```

- Add new columns into the database

```
var col =
  table.insertUserColumn("","Value");
for (row in table)
  table[row][col]
    = table[row][1]/table[row][2];
```

- Export tables using CsvWriter

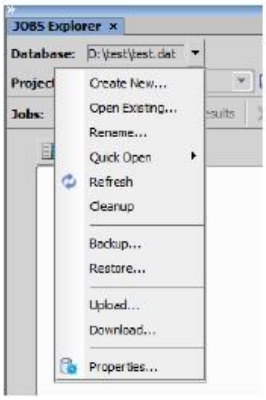
```
var f = new CsvWriter(filename, false);
f.writeValues(colNames);
f.writeValues(colValues);
f.close();
```

For more details see the JS Documentatuion.

The script can be saved and reused for similar classifications.

Backing up data

Full database (by default with all its files) is backed up by command “Backup…” from the main JOBS menu and from the Database menu in JOBS Explorer.



This form of backup does not allow incremental or partial backups. It is a one-to-one copy.

The inverse operation is a Restore. It copies the contents of some foreign database into the current one.

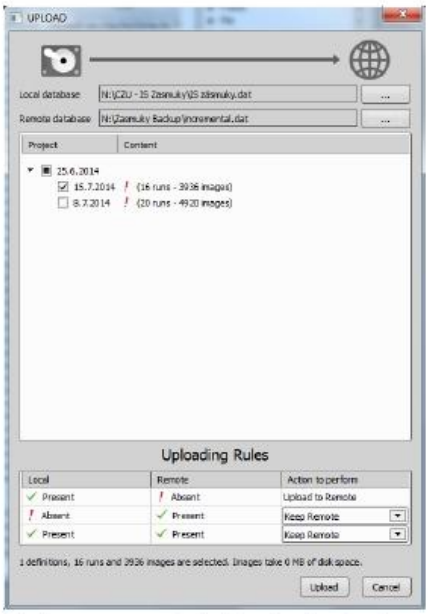
NOTE: It is not safe to simple copy the Folder with the database!

Uploading database

Instead of doing complete backup, it is more convenient to do incremental (only what have changed is uploaded) backups using Upload.

In the dialog it is possible select what to upload (the software is showing incomplete jobs on in the remote location) and what rules to apply to possible conflict:

- “Keep Remote” (default) does not delete anything – keeps adding to remote.
- “Delete from remote” deletes records to synchronize with the local state.
- “Overwrite using local” uses the local state to update remote in case of conflict.



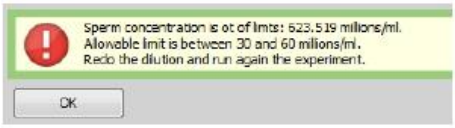
The inverse operation is Download, whereby similarly only a portion of some remote database is downloaded on the local computer.

Improving JOB

Adding concentration check

Job definition can be easily set to check some sample parameters and give the operator some hints.

In this example case the sperm concentration went out of limits and the operator is instructed to redo the dilution.



Here is the Job definition which does it (the part in the rectangle):

It captures one image and counts sperms in the whole frame. It then uses the formula

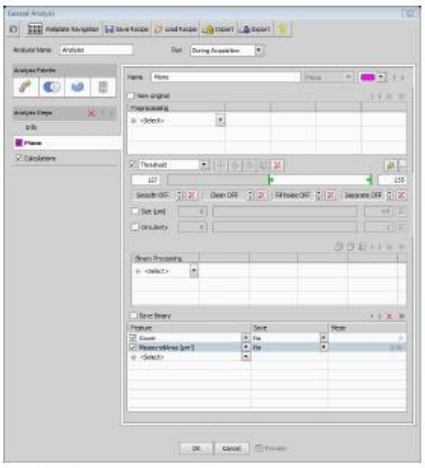
$$Concentration = \frac{(500\ 10^{8} ObjectCount)}{MeasuredArea}$$

to calculate the concentration of sperms as number/ml. This value is then checked in the "If" Task

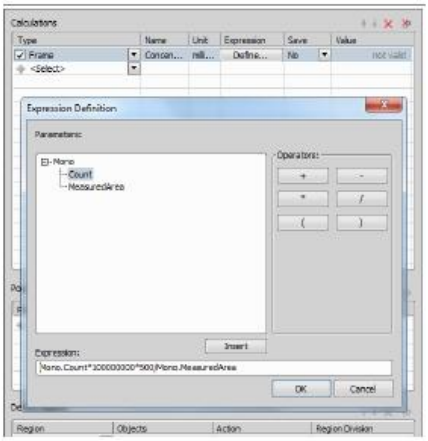
…and the job can then be aborted if it fails in the check and has to be prepared again.

Here is how to setup the General Analysis:

1. Add one Channel Tab (default name is taken from the single component).
2. Set the threshold and add post-processing and restrictions (as described in Running Analysis section) in order to get desired segmentation.
3. Uncheck "Save binary".
4. Add two measurement features: Count and MeasuredArea.
5. Set "No" in the Save column as these are only temporary.
6. Add Calculations tab

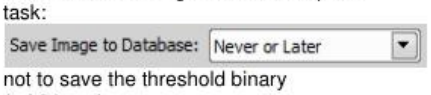


7. Set the
   - Type to "Frame",
   - Name to "Concentration"
   - Unit to Number/ml
   - Save to "No"
8. In the Define set the formula using the tree of available features (Count and MeasuredArea) and click Ok.



NOTE: As this check should not add data to the resulting dataset it is important to:

- not to save the image inside the capture task:



- not to save the threshold binary (ad 3.) and
- not to save the measurement results (ad 5. and ad 7.)

Running the SpermMotility analysis automatically after acquisition

Any user defined action can be done after JOB finishes the run.

In the following example:

1. an analysis recipe “SpermMotility” (last argument) is run to create “SpermMotility” (second argument) analysis using a macro function “Jobs_RunAnalysis(…)” (see the help for details).
2. a JavaScript is executed on analyzed data to show the motility in the acquired sample.

Workflow

A. Create a new DB

From saved files

1. Import Custom Metadata (menu):
   JOBS → Manage Custom Metadata…
   

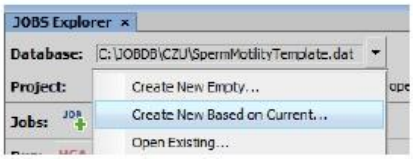
2. Create New Project (JOBS Explorer)
   

3. Import Job definition (JOBS Explorer)
   

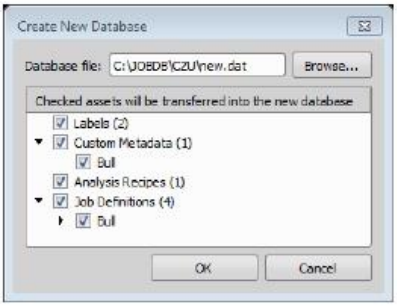
4. Import Tracking recipes (menu)
   JOBS → Manage Recipes…
   

From existing database

1. Select "Create New Based on Current…" from the Database popup menu or the main menu.



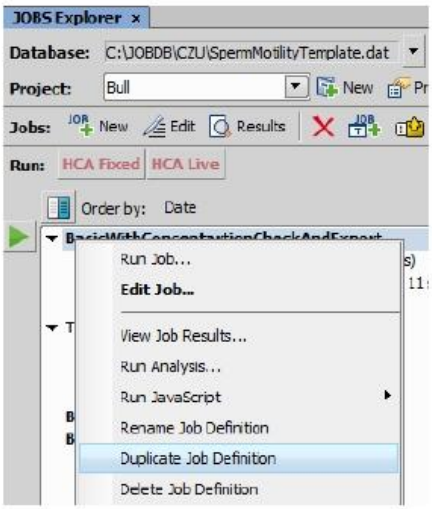
2. Check the items to copy into the new database.



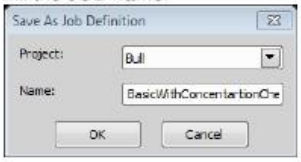
B. Create new JOB (experiment day)

Duplicating a JOB is the best way to ensure consistency (same definition) across experiments.

1. Choose a JOB you want to copy and select "Duplicate Job Definition".



2. Fill the JOB name.



NOTE: It is good idea to keep some JOBs empty just as a template for duplicating. The may be a separate project (e.g. Templates).

3. Run the experiments

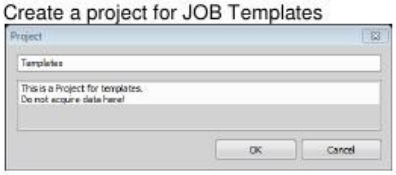
C. Uploading/Downloading

There is often limited disk space on the acquisition computer and many users running their experiments. In such an environment the best practice is that every user manages his experiment data.

It can be done with portable disk in such a way that tha used space on the experiment machine is limited to few experiments.

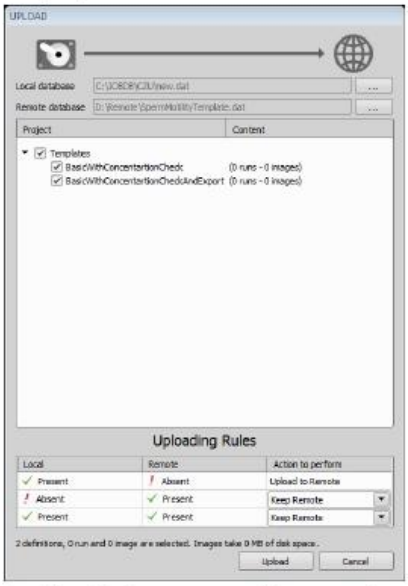
First Time (setup)

1. Creates a new database on the experiment machine in (your) working folder as described above.
2. Create a project for JOB Templates



3. Import, Create or Duplicate Job Definitions into the Templates prooject.

4. Upload the Database onto you portable disk. Set the remote Database to point to your portable disk.



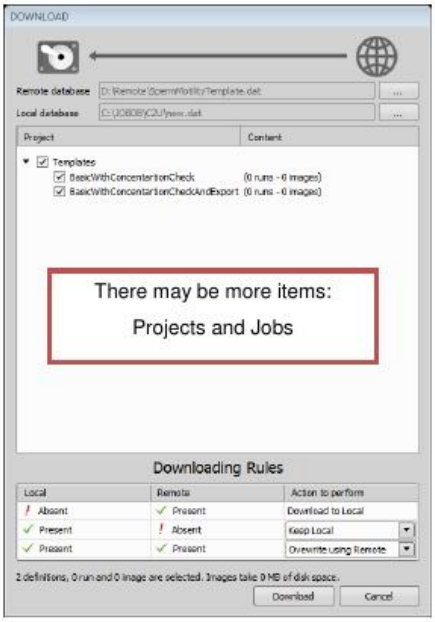
5. After uload a message with success is diplayed.

After every experiment day (in the office)

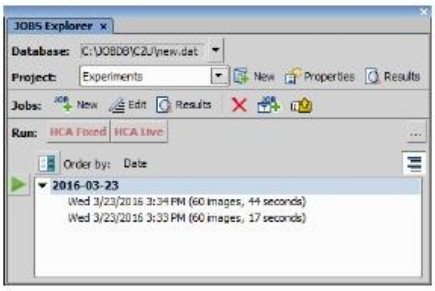
1. Connect the portable disk and backup the database (with images) to another computer. If there is no NIS Elements installed just copy the files.
2. Review or ananalyze the data.

Before every experiment day

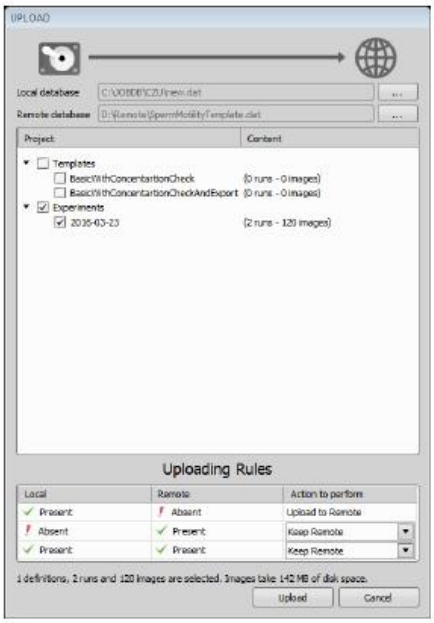
1. Attach the portable disk to the experiment machine. There may still be the database from the last experiment day (or setup) in (your) working folder.
2. In case it was deleted create a new one from the menu “Create New Empty…” in (your) working folder.
3. If empty database was created or you modified the templates or metadata Download the database from the portable disk, selecting the portable disk as Remote.



4. It is important to download only the Templates project and not the data. Select “Overwrite using Remote” to updated metadata.
5. Create New Project if necessary (there is only one project – “Templates”) in order not to acquire into Templates.
6. Create a new JOB by Duplicating a template. Set the date as name of the JOB. Don’t forget to change the project.
7. Acquire several Jobruns.



8. Upload acquired JOB and jobruns to the portable disk.



The Database on the portable disk will grow.

REMEMBER to backup the database on the portable dist to another disk (in the ofice at home).

Cleanup on the experiment machine

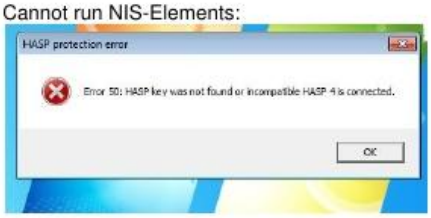
The users working folders should be cleaned up from time to time to reclaim disk space. The period should be long enough to leave some time for users to backup their data and short anough to have enough space disk space for safe acquisition.

Troubleshooting

- Insufficient disk space:

  Backup (using the appropriate menu function) the experiments to the external or other disk.

- Cannot run NIS-Elements:



  Reinsert the HASP (USB) dongle.

- Problems with camera connection:

  Reinsert the USB camera cable.