UNIVERZITA KARLOVA V PRAZE

Přírodovědecká fakulta

DISERTAČNÍ PRÁCE

2018 MVDr. Božena Bohuslavová

Univerzita Karlova v Praze

Přírodovědecká fakulta

Vývojová a buněčná biologie

MVDr. Božena Bohuslavová

Fenotypová štúdia Huntingtonovej choroby TgHD miniprasiat: Nástup a priebeh reprodukčných zmien

Phenotypic study of Huntington's disease TgHD minipigs: Appearance and progress of reproductive and biochemical changes

Dizertačná práca

Školiteľ: prof. MVDr. Jan Motlík, DrSc.

Praha, 2018

Prehlasujem, že som záverečnú prácu spracovala samostatne a že som uviedla a citovala všetky použité informačné pramene a literatúru. Súčasne prehlasujem, že táto práca ani jej podstatná časť nebola predložená k získaniu iného alebo rovnakého akademického titulu.

Dizertačnú prácu som vypracovala na Ústavu živočišné fyziologie a genetiky Akademie věd České Republiky, v.v.i. v Liběchově.

V Prahe, dňa .....

..... Božena Bohuslavová

V prvom rade by som sa chcela poďakovať školiteľovi prof. MVDr. Janovi Motlíkovi, DrSc. za podporu a ľudský prístup v priebehu môjho doktorandského štúdia. Taktiež veľké ďakujem patrí mojim všetkým kolegom z Laboratoře buněčné regenerace a plasticity ÚŽFG AV ČR, v.v.i. v Liběchově za cenné rady, pomoc a príjemnú pracovnú atmosféru. Zvlášť ďakujem Ing. Zdenke Ellederovej, PhD., MVDr. Štefanovi Juhásovi, PhD. Ďakujem svojej rodine a priateľom za ich podporu a trpezlivosť.

Práca vznikla za finančnej podpory americkej nadácie CHDI (A-8248, A-5378), projektu ExAM (CZ.1.05/2.1.00/03.0124), výskumného zámeru ÚŽFG AV ČR, v.v.i. (RVO 67985904), Finanční mechanismy EHP/Nórsko (7F14308),

ABSTRAKT

Huntingtonova choroba (HCH) patrí k nevyliečiteľným ochoreniam s fatálnym koncom. Zaraďujeme ju k monogénnym neurodegeneratívnym ochoreniam. Podľa počtu opakovaní CAG repetícii v géne kódujúcom huntingtín, nástup ochorenia môže byť v detskom veku (5%), strednom veku – najčastejšie (90%) a vyššom veku (5%). Začiatok choroby sa prejaví zmenami v správaní, ku ktorým sa pridružia problémy s koordináciou a pohybom. Neskôr dochádza k psychickým zmenám. Ochorenie končí smrťou. V dnešnej dobe zatiaľ nie je vyvinutá účinná liečba. Doktori momentálne len príznaky farmakologicky utlmujú, ale nelieči sa príčina choroby.

Na našom ústave v Liběchove sme v roku 2009 vytvorili model transgénneho miniprasaťa (TgHD) nesúceho N - terminálnu časť ľudského mutovaného huntingtínu (mtHtt). Dostatok potomkov a veľká príbuznosť fyziológie a morfológie medzi prasaťom (*Sus scrofa)* a človekom *(Homo sapiens)* nám dáva dostatok možnosti na študovanie zmien v centrálnych nervových orgánoch, ale i v periférnych tkanivách. Ako prvé zmeny fenotypu sme pozorovali reprodukčné problémy TgHD kancov. Preto sa moja práca v prvom rade zamerala na komplexné preštudovanie reprodukčných parametrov TgHD kancov, rovnako ako na ultraštrukturálne, imunocytochemické a biochemické zmeny v tkanivách semenníkov a v spermiách.

V tejto práci som detailne popísala reprodukčné defekty u TgHD kancov F1 a F2 generácie. Všetky získané výsledky boli vždy porovnávané medzi netransgénnymi (WT) a transgénnymi kancami (TgHD) rovnakého vrhu. U obidvoch generácii TgHD kancov bol zistený nástup reprodukčných zmien vo veku 13tich mesiacov. Zmeny v počte spermií, v ich pohybe a v progresívnom pohybu dopredu za hlavičkou vykazovali signifikantné rozdiely vo veku 24 až 36 mesiacov. Transmisná elektrónová mikroskopia preukázala početné morfologické abnormality predovšetkým v oblasti bičíku spermií a taktiež v testikulárnom epiteli vo veku 24 a 36 mesiacov. Tieto morfologické pozorovania boli podopreté imunocytochemickým sledovaním, ktoré preukázalo signifikantne nižšiu proliferačnú aktivitu spermatogónií TgHD kancov. Zásadné zistenie predstavuje vysoká expresia mutovaného huntingtínu (mtHTT) vo všetkých bunkách semenotvorných kanálikoch semenníkov TgHD kancov a taktiež vo všetkých častiach bičíka ejakulovaných spermií, predovšetkým v ich spojovacích častiach. Tieto zmeny boli taktiež potvrdené neinvazívnym prístupom, vyšetrením testikulárneho tkaniva pomocou ^{31}P magnetické spektroskopie (MRS)

u súrodencov (WT a TgHD kancov). I tento prístup preukázal signifikantný pokles v pomere fosfodiesterov (PDE/γ-ATP) v parenchýme semenníkov TgHD kancov.

Vyššie popísané zmeny v spermiách na úrovni morfológie a funkcie boli zásadným spôsobom potvrdené pomocou detailného štúdia metabolizmu mitochondrií u spermií WT a TgHD kancov. Hlavnou výhodou tohto biologického materiálu je možnosť jeho neinvazívneho získavania v dlhšom časovom intervale. Funkcie dýchacieho reťazca boli študované pomocou polarografie a potom bol metabolizmus mitochondrií stanovený pomocou detekcie oxidácie rádioaktívnych substrátov. Tento metodických prístup preukázal signifikantný pokles v dýchacom komplexe I a taktiež v štyroch parametroch, ktoré indikujú významné zníženie úrovne glykolytickej dráhy v spermiách TgHD kancov. Tieto experimenty detailne charakterizovali biomarkery pre monitorovanie rozvoja HCH. Predložené výsledky jednoznačne potvrdili negatívni vplyv mutovaného huntingtínu na mitochondriálny metabolizmus, ktorý vedie k degenerácii parenchýmu semenníkov a k zásadným zmenám vo funkcii spermií.

Všetky experimenty zaradené do tejto dizertačnej práce potvrdili, že TgHD miniprasaťa, u ktorých bol preukázaný postupný rozvoj predklinického fenotypu HCH, sú vhodným, biomedicínskym modelom pre testovanie účinnosti všetkých súčasných prístupov k ovplyvneniu rozvoja HCH.

ABSTRACT

Huntington's disease (HD) is one of the incurable and fatal diseases. HD belongs to the monogenic neurodegenerative diseases. According to the number of the CAG repetitions in the gene coding huntingtin, the onset of the disease is in childhood (5%), in the middle age, which is the most common (90%) and in the older age (5%). Beginning of the disease is manifested by changes in behavior; including problems with coordination and movement. Later, there is a psychological change. The disease leads to death. Until now, there is no effective curative treatment.

In 2009, we created a model of the transgenic minipigs (TgHD) carrying the N - terminal part of the human mutant huntingtin (mtHtt) at our Institute in Liběchov. The number of offsprings and the resemblance in physiology and morphology between the pig (*Sus scrofa*) and humans (*Homo sapiens*) give us opportunities not only to study changes not only in central nerve organs, but also in peripheral tissues. The reproductive problems of TgHD boars were observed as the first phenotypic changes. Therefore, my work focuses at first on a study of the reproduction parameters of TgHD boars as well as ultrastructural, immunocytochemical and biochemical changes in testes and spermatozoa.

In PhD thesis, I described in details the reproductive defects in TgHD boars of F1 and F2 generations. All gained data were always compared between the wild-type (WT) and TgHD boars from the same litter. The reproductive changes started at the age of 13 months in both generations of TgHD boars. Significant changes in number of spermatozoa per ejaculate, in their movement and in their progressive movement were confirmed at the age of 24 and 36 months, too. Transmission electron microscopy (TEM) revealed numerous morphological abnormalities, first of all at sperm tails and testicular epithelium at the age of 24 and 36 months. These morphological observations were supported by immunocytochemical approaches that confirmed the significantly lower proliferation activity in spermatogonia of TgHD boars. The key result is that the high expression of mtHTT occurs in all cells of the seminiferous tubules and also in all parts of sperm tail, in the midpiece especially. The described changes were also confirmed by the noninvasive approach, using ^{31}P magnetic spectroscopy (MRS) for examination of testicular tissue of siblings (WT and TgHD boars). The significant decrease in ratio of phosphodiesterases (PDE/γ-ATP) in the testicular parenchyma of TgHD boars was documented.

All morphological and functional changes described above were confirmed in the detailed study of mitochondrial metabolism in spermatozoa of WT and TgHD boars. The key advantage of this biological material is that it can be collected noninvasively and longitudinally. The oxidative function was measured by polarography and metabolism of mitochondria was also measured after oxidation of radioactive substrates. These methodological approaches proved the significant decrease in the activity of respiratory complex I and they also revealed four parameters indicating the serious impairment of glycolytic activity in spermatozoa of TgHD boars. These experiments described in details biomarkers monitoring progress of HD and fully confirmed the negative effect of mtHTT on mitochondrial metabolism leading to testicular degeneration and to substantial functional changes of spermatozoa. .

The experiments in this PhD thesis clearly demonstrated that TgHD minipigs, with the preclinical development of the HD phenotype, represent the suitable, biomedical model for testing of all current methods of gene therapy of HD.

OBSAH

1. ÚVOD 12
1.1. George Huntington 13
1.2. Huntingtonova choroba 13
1.2.1. Funkcie a štruktúra proteínu huntingtínu 18
1.2.2. Patológia orgánov pri HCH 20
1.2.2.1. Patológia mozgu 20
1.2.2.2. Patológia kardiovaskulárneho systému 20
1.2.2.3. Patológia kostrového svalu 21
1.2.2.4. Testikulárna patológia 21
1.2.2.5. Správanie a zmeny správania pri Huntingtonovej chorobe 21
1.3. Terapia Huntingtonovej choroby 22
1.3.1. terapia Huntingtonovej choroby pomocou génovej terapie 23
1.4. Zvieracie modely Huntingtonovej choroby 24
1.4.1. Liběchovský model transgénneho miniprasaťa pre HCH 25
1.5. Zloženie ejakulátu kancov 26
1.5.1. Stavba a funkcia spermií 26
1.5.2. Spermiogenéza 27
1.6. Patológia ejakulátu 30
2. CIELE 32
3. METÓDY 33
3.1. Experimentální model 33
3.2. Odber semena a testy na určenie parametrov semena 33
3.3. Príprava spermií a testikulárneho tkaniva pre imunohistochémiu a Western blot 38
3.4. Príprava vzoriek pre hormonálnu analýzu 39
3.5. Príprava spermií pre transmisnú elektronovú mikroskopiu, vyhodnotenie vzoriek 39
3.6. Príprava kancov pre 31P MR spektroskopiu 40

3.7. Príprava spermií pre štúdium metabolizmu mitochondrií 41
4. VÝSLEDKY 46
4.1. Mutovaný huntingtín spôsobuje testikulárnu patológiu u kancov transgeného miniprasaťa 47
4.2. ^{31}P MR spektroskopia semenníkov a imunohistochemická analýza spermií transgénnych kancov realizovaná pre N koncovú časť ľudského mutovaného huntingtinu 55
4.3. Mitochondriálny metabolizmus na veľkom zvieracom modely pre Huntingtonovú chorobu: hľadanie biomarkerov v spermiách presymptomatických miniprasiat..... 59
5. DISKUSIA 66
6. ZÁVER 70
7. ZOZNAM CITOVANEJ LITERATÚRY 71
8. PREHĽAD VŠETKÝCH RUKOPISOV V RÁMCI PhD. ŠTÚDIA 89
8.1. Publikácie v impaktovaných časopisoch 89
8.2. Publikácie v knižných zborníkoch 90
8.3. Publikácie v neimpaktovaných časopisoch 90
8.4. Prezentácie prostredníctvom posterov 91
9. PREHĽAD PRÍLOH 95
10. CURRICULUM VITAE 96

ZOZNAM SKRATIEK

AAV5	*Adeno – Associated Virus serotyp 5*
AMK	Aminokyselina
ANS	Autonómny nervový systém
ASOs	Antisentense oligonukleotidy
ATB	Antibiotika
BDNF	(z angl. **b**rain-**d**erived **n**eurotropic **f**actor).
CAG	Cytozín-Adenín-Guanín, kód pre amynokyselinu glutamín
CNF	Centrálna nervová sústava
CSF	Cerebro spinálna tekutina
DNA	deoxy-nukleová kyselina
dsDNA	double-stranded DNA, dvojvláknová DNA
ER	Endoplazmatické retikulum
FSH	Folikulo-stimulačný hormón
GA	Golgiho aparát
GABA	gama-aminomaslová kyselina
GDNF	Glial cell-Derived Neurotrophic Factor – neurotrofický faktor pochadajúci z gliálnej línie
GFP	Green fluorescent protein, zelený florescenčný proteín
GGT	Gama Glutamyl Transferáza
GPC	Glycerofosfocholín
GPE	Glycerofosfoetanolamín

HEAT	z domény z angl. Huntingtin, Elongator factor3, the regulatory A subunit of protein phosphatase 2A, and TOR1
HCH	Huntingtonova choroba
HTT	Huntingtín
IL8	Interleukín 8
IL 10	Interleukín 10
INF-α	Interferón α
MEGS	mitochondriálny systém generujúci energiu
Mg^{2+}	horečnatý katión
mtHtt	mutovaný huntingtín
miHtt	mikro huntingtín
miRNA	mikro RNA
MM	mitochondriálny metabolizmus
MRI	z angl. *Magnetic Resonance Imaging*
mRNA	messenger RNA
NES	z anglického nuclear export signal
NLS	z anglického nuclear localization signal
OXPHOS	oxidatívna fosforylácia
PCr	fosfokreatinín
PDE	fosfodiester
PET	z angl. *Positron Emission Tomography*
PDHc	pyruvát dehydrogenázový komplex
Pi	inorganic phosfate – anorganický fosfát

PME	fosfomonoester
polyQ	polyglutanímový reťazec
RISC	*RNA-induced silencing complex*
RNA	ribonukleová kyselina
RNAi	RNA interferencia
SDHA	sukcinát dehydrogenáza A
siRNA	small interfering RNA, malá interferujúca RNA
ssDNA	single strended DNA, jednovláknová DNA
ssRNA	single strended RNA, jednovláknová RNA
TgHD	transgénne miniprasa pre Huntingtinovu chorobu
TNF-α	Tumor necrosis factor α
WT	*Wild-Type*
ZFPs	zinc-fingers proteíny

ZOZNAM OBRÁZKOV

Obr.č. 1 **George Huntington a jeho článok v The Medical and Surgical Reporter** 13
Obr.č. 2 **Domény huntingtínu a miesta postranslačných úprav** 19
Obr.č. 3 **Štúdie génovej terapie** 23
Obr.č. 4 **Prvý vrh prasiat vzniknutých z injektovaných embryí** 25
Obr.č. 5 **Stavba spermie** 27
Obr.č. 6 **Spermihistogenéza** 29
Obr.č. 7 **Spermie s cytoplazmatickou kvapkou** 29
Obr.č. 8 **Teratospermia** 31
Obr.č. 9 **Sperm cell analyzer** 34
Obr.č. 10 **Zloženie roztokov používaných pri penetračných testoch** 37
Obr.č. 11 **Pozície spektroskopickej mriežky v programe jSIPRO** 41
Obr.č. 12 **Plán odberu vzoriek pre meranie MEGS** 42
Obr.č. 13 **plánodberu vzoriek pre respiračné analýzy** 42
Obr.č. 14 **Počet spermií a motilita** 48
Obr.č. 15 **Počet spermií** 49
Obr.č. 16 **Motilita spermií**. 49
Obr.č. 17 **Progresivita spermií** 50
Obr.č. 18 **Znížená penetračná schopnosť spermií** 50
Obr.č. 19 **Spermia s cytoplazmatickou kvapkou a bičík s trojitou axonémou** 51
Obr.č. 20 **Semenotvorný epitel TgHD kancov vo veku 36 mesiacov**. 52
Obr.č. 21 **Expresia Htt v semenníkoch**. 53
Obr.č. 22 **Distribúcia pomerov PDE/γ-ATP, PME/γ-ATP and Pi/γ—ATP u TgHD kancov a ich Wt súrodencov** 56
Obr.č. 23 **Imunofluorescenčné farbenie spermií TgHD a WT kancov z F1 a F3 generácie** 57
Obr.č. 24 **Kvantifikácia florescenčného signálu v spermiáchfarbených EPR5526** 57
Obr.č. 25 **Lokalizácia mitochondrií a proteínov**. 61
Obr.č. 26 **Inkubácia MEGS s [1-14C] pyruvátom + karnitínom + ADP** 62
Obr.č. 27 **Systém mitochondriálnej energie (MEGS) a respiračné parametre** g 63

Obr.č. 28 **(a) Analýza vybraných podjednotiek oxidačného fosforylačného systému (OXPHOS) a komplexu pyruvátdehydrogenázového komplexu (PDHc) u TgHD kancov**; 64
Obr.č. 29 **Katalytická aktivita oxidačných fosforylačných komplexov (OXPHOS) u spermií TgHDkancov vo veku 14 mesiacov.** 65

1. ÚVOD

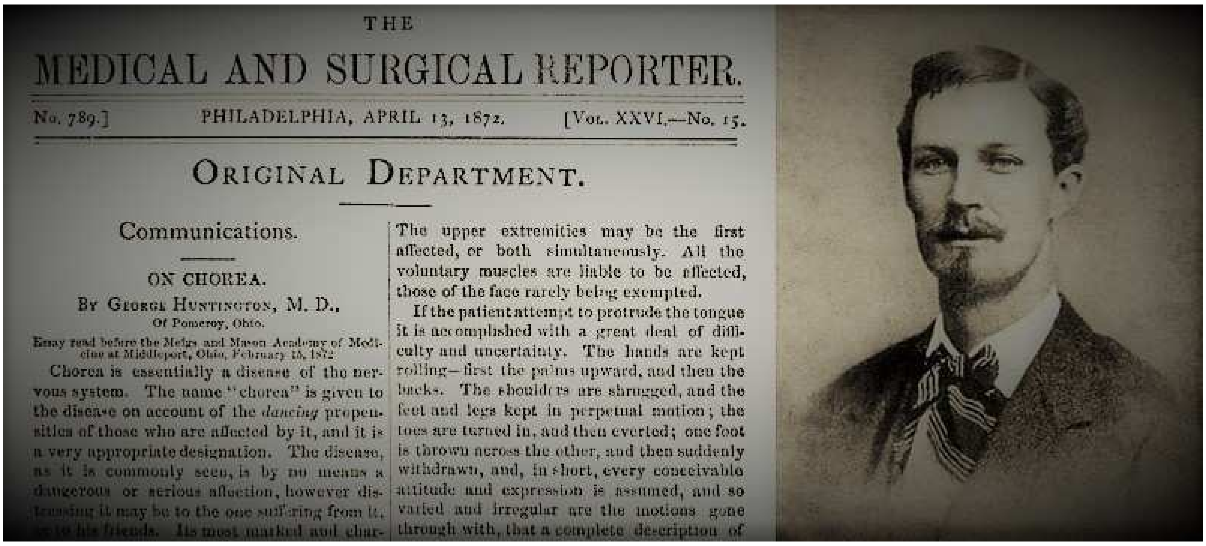
Huntigtonova choroba (HCH) je neuorodegeneratívne dedičné ochorenie, s progresívnym priebehom, končiace fatálne. Prevalencia v strednej Európe je približne 5 prípadov na 100 000 obyvateľov. Predpoklad prvotnej patológie HCH bola degenerácia bazálnych ganglií (prejavy ako zmeny v správaní, znížená koordinácia). Vedci dokázali expresiu mutovaného huntingtínu (mtHtt) vo všetkých študovaných tkanivách. Ochorenie spôsobuje zmnoženie CAG repetícii v géne kódujúcom huntingtin (HTT).Prvý krát túto chorobu opísal George Huntington. Spolu s otcom dlhodobo pozorovali dedičné prejavy ochorenia vo viacerých generáciách v rodine bývajúcej v East Hampton. Najprv u nich začínali zmeny v pohybe, čo nazval Huntington ako tanečná mánia. Po vypuknutí týchto príznakov choroba do 10 rokov končila smrťou.

V roku 2009 sa na AV ČR v Liběchove vytvoril model transgénneho miniprasaťa pre HCH (Baxa et al., 2013). Ako prvé sa prejavili zmeny a reprodukčnom aparáte samcov. Vedecký poznatok, že mtHtt má expresiu i v tkanivách ne-neurálneho pôvodu a súčasnom prebiehaní prasničiek, pripúšťaných našimi transgennými (TgHD) kancami, sme si položili otázku, či to nemôže mať súvis s HCH. Podľa Gua (Guo et al., 2003) práve semenníky majú najviac porovnateľnú génovú expresiu s mozgom spomedzi všetkých periférnych orgánov. Poruchy spermiogenézy a abnormality testes boli pozorované ako u transgénnych modelov myší tak i ľudských pacientov.

Cieľom dizertačnej práce bolo zamerať sa na identifikáciu a priebeh reprodukčných zmien TgHD kancov. V prvom rade som sa zamerala na dôkladne preštudovanie reprodukčných parametrov TgHD kancov. Pozorovala som imunocytochemiké a morfologické zmeny spermií. Pomocou neinvazívnej metódy ^{31}P magnetickej spektroskopie sme chceli preukázať zmeny v semenníkoch. Ďalšou úlohou bolo dôkladné preštudovanie metabolizmu mitochondrií v spermiách. Vzhľadom na veľkú fyziologickú podobnosť medzi človekom (*Homo Spaiens*) a prasaťom (*Sus Scrofa*) by model TgHD miniprasaťa pre HCH mohol byť vhodný k určeniu biomarkerov a následnému štúdiu možnej terapie, ktorá by mohla prispieť k skorému klinickému skúšaniu terapie na ľudských pacientoch.

1.1. George Huntington

George Huntington (Apríl 9, 1850 – Marec 3, 1916) pochádzal z rodiny lekárov. Zaslúžil sa o preštudovanie choroby, ktorá dnes nesie jeho meno – Huntingtonova choroba. Vo veku 22 rokov, po ukončení lekárskej fakulty v Kolumbii zverejnil svoju prácu v *The Medical and Surgical Reporter* (13.4.1872). Popisoval v nej dedičnú formu chorey. Jeho publikácia sa stala jedným z klasických popisov neurologického ochorenia (Neylan, 2003).



THE

MEDICAL AND SURGICAL REPORTER.

No. 789.] PHILADELPHIA, APRIL 13, 1872. [Vol. XXVI.—No. 15.

ORIGINAL DEPARTMENT.

Communications.

ON CHOREA.

By George Huntington, M. D.,
Of Pomeroy, Ohio.

Essay read before the Meigs and Mason Academy of Medicine at Middleport, Ohio, February 15, 1872

Chorea is essentially a disease of the nervous system. The name "chorea" is given to the disease on account of the *dancing* propensities of those who are affected by it, and it is a very appropriate designation. The disease, as it is commonly seen, is by no means a dangerous or serious affection, however distressing it may be to the one suffering from it, ... his friends. Its most marked and char-

The upper extremities may be the first affected, or both simultaneously. All the voluntary muscles are liable to be affected, those of the face rarely being exempted.

If the patient attempt to protrude the tongue it is accomplished with a great deal of difficulty and uncertainty. The hands are kept rolling—first the palms upward, and then the backs. The shoulders are shrugged, and the feet and legs kept in perpetual motion; the toes are turned in, and then everted; one foot is thrown across the other, and then suddenly withdrawn, and, in short, every conceivable attitude and expression is assumed, and so varied and irregular are the motions gone through with, that a complete description of

Obr.č. 1 ***George Huntington a jeho článok v The Medical and Surgical Reporter*** *(upravené zo zdroja File:On Chorea with photo.jpg - Wikimedia Commons)*

1.2. Huntingtonova choroba

Predtým, než Huntingtonova choroba dostala svoj názov po Georgovi Huntingtonovi (Huntington, 2003; Neylan, 2003), sa pre toto ochorenie od 14. storočia používal názov „tanečná mánia“. Ide o autozomálne dominantne dedičné ochorenie, s neurodegeneratívnymi príznakmi a progresívnym priebehom. Vo väčšine prípadov sa prejaví v dospelosti, len asi 5 % prípadov v detskom veku (Roth, 2010). Typické príznaky sú choreatické pohyby, zníženie mentálnych schopností. Priebeh je progresívny s fatálnym koncom. V roku 1983 bol identifikovaný polymorfný DNA marker, lokalizovaný na ľudskom chromozóme 4,

signalizujúci HCH (James F. Gusella et al., 1983). V roku 1993 bol objavený jediný kauzálny gén, ktorého mutácia je zodpovedná za HCH (J F Gusella, Persichetti, & MacDonald, 1997)

Riziko choroby podľa počtu opakovania tripletu CAG

10 – 26 CAG	jedinec je zdravý, bez choroby (normálny počet u zdravého jedinca) (Walker, 2007)
27 – 35 CAG	ak nevznikne mutácia, jedinec je zdravý, pri náhodnej mutácii prejavujúcej sa zmnožením tripletu, môže dôjsť k ochoreniu
36 – 41 CAG	pacienti majú Huntingtonovu chorobu, prejavujúca sa v starobe (Rubinsztein et al., 1996)
40 – 55 CAG	prejav ochorenia je viditeľný okolo 40 roku života (de Die-Smulders, de Wert, Liebaers, Tibben, & Evers-Kiebooms, 2013; Montoya, Price, Menear, & Lepage, 2006)
≥ 60 CAG	prejavy začínajú už v detstve (Walker, 2007)

Formy Huntingtonovej choroby

Klasická – začína obyčajne medzi 35 – 50 rokom života (cca 90 % všetkých prípadov)

Juvenilná – začiatok do 20 roku života (cca 5%)

Forma s neskorým začiatkom – začiatok po 60 roku života (cca 5%)

Klasická forma

Prvé príznaky sa objavujú medzi 35 – 50 rokom života. Počiatočné problémy sú nešpecifické – poruchy chovania, zmeny povahy, môžu sa vyskytnúť časté depresívne stavy. V neskoršom štádiu sa prejaví demencia. V začiatočnom štádiu sa objavujú mimovoľné pohyby a porucha

cielených pohybov. V neskoršom štádiu dochádza k neistote pri chôdzi, poruchám reči , taktiež môže nastať problém s prehĺtaním , čo vyústi k následnej kachekcii. V pokročilom štádiu ubudne mimovoľných pohybov. Priebeh choroby je individuálny, ale v priemere pacient v priebehu 15 rokov sa stáva závislým na starostlivosti okolia. Umiera v nezadržateľnom marazme, ku ktorému sa väčšinou pričlení infekcia a iné druhotné komplikácie (Zelená,2009; Roth, 2010).

Juvenilná forma

Začína pred 20. rokom života. Jej prevalencia je približne 5% (Rasmussen et al., 2000) z celkového počtu všetkých pacientov postihnutých Huntingtonovou chorobou. Dokonca sa môže stať, že sa choroba prejaví pred 10 rokom (0,5-1% všetkých prípadov). Typický začiatočný prejav je nezvládanie školských aktivít (dôsledok kognitívnej poruchy a motorického spomalenia) (Roth, 2012). Jasným príznakom je dyskoordinácia pohybu. Pozorujeme poruchy správania sa – výbuchy zlosti, agresivitu, antisociálne správanie (Ribaï et al., 2007). Rigidita, dystónia a hypokinézia sú hlavnou príčinou rýchle progredujúcej poruchy stability a chôdze. Mimovoľné pohyby sa obvykle neobjavia. Asi u 30% postihnutých jedincov sa objavia epileptické záchvaty (Rasmussen et al., 2000; Siesling, Vegter-van der Vlis, & Roos, 1997). Dochádza k poruchám prehĺtania a výslovnosti. Rýchla progresia vedie k demencii a k nesamostatnosti, úplná závislosť na starostlivosti okolia (Roth 2012).

Forma s oneskoreným začiatkom

Prevalencia je okolo 5%. Jej prvotné príznaky sa začnú objavovať okolo 60. roku života. Veľmi výnimočné je pozorovanie prvých príznakov až po dovŕšení sedemdesiatky. Táto forma má pomalý priebeh, a pacienti sa dožívajú bežného priemerného veku zdravej populácie. Hlavným príznakom sú mimovoľné pohyby. Obyčajne nespôsobujú závažné postihnutie základných denných aktivít a pacienti sú po motorickej stránke sebestačný. Výraznej demencii obyčajne nedôjde (Lipe & Bird, 2009).

Príznaky Huntingtonovej choroby

Neurologické	porucha koordinácie hybnosti – huntingtonická chorea od ktorej sa odvíjajú nasledovné príznaky: poruchy chôdze poruchy reči poruchy prehĺtania (môže podmieňovať následné chudnutie)

Psychické	zmeny povahy a poruchy správania: apatia alebo naopak predráždenosť (Jason et al., 1997) nezvládanie bežných aktivít agresivita drobná kriminalita (J S Paulsen, Ready, Hamilton, Mega, & Cummings, 2001) zmeny sexuálneho chovania poruchy nálady – predovšetkým depresia úzkosť (Snowden, Craufurd, Griffiths, Thompson, & Neary, 2001)
Psychotické prejavy	bludy halucinácie porucha pamäti, sústredenia, demencia
Reprodukčné	znížená reprodukčná schopnosť (tieto príznaky boli zistene u modelových zvierat – laboratórnych myší R6, YAC 128: (Papalexi et al., 2005; Van Raamsdonk et al., 2007) znížený počet spermií znížená životaschopnosť spermií celková testikulárna degenerácia, zistená na posmrtných vzorkách u mužských pacientov.

Neurologické prejavy

Najcharakteristickejší hybný prejav choroby je huntingtonická chorea – mimovoľné, nepravidelné najčastejšie šklbavé pohyby tváre, končatín a trupu (Vonsattel et al., 1985, Kosinski et al., 2005). Chôdza je kolísavá, môže sa zdať, že má tanečný ráz. Tieto mimovoľné pohyby sa v priebehu choroby zhoršujú, v konečných fázach choroby tieto pohyby nemusia byť viditeľné, svalstvo je stuhnuté a pacient sa stáva nepohyblivým (Ross., 2002).

Poruchy chôdze – rozvoj približne v strede ochorenia. Častá je chôdza na široko rušená mimovoľnými pohybmi. V neskorších fázach ochorenia dochádza k častým pádom pri chôdzi, pacienti sú v priestore neistý.

Poruchy reči – prejav v strednej a neskoršej fáze ochorenia. Postupom času progreduje a reč sa stáva nezrozumiteľnou. Reč je charakteristická explozívna a sakadovaná (zvýrazňovanie niektorých slabík). Môžeme pozorovať mimovoľné zvuky ako bučanie, povzdych.

Poruchy prehĺtania – neskoré štádia choroby. Vznikajú nekoordinovaným pohybom hltanového svalstva. To vedie k závažným poruchám príjmu potravy, možnosti udusenia, následnému chudnutiu (chuť do jedla je väčšinou zachovaná).

Psychické prejavy

Rodina tieto prejavy popisuje ako zmeny osobnosti a chovania. Často dochádza k zníženej vnímavosti voči potrebám druhých, k zníženému záujmu o prácu, o vlastný zovňajšok, náladovosť a zlyhávanie v bežných aktivitách (Duff et al., 2010; Rosenblatt, 2007).

Podráždenosť – je vyprovokovaná okolnosťami, ktoré by inokedy takúto odpoveď nevyvolali. Intenzita a trvanie takejto odpovede nie je úmerná okolnostiam, často dochádza k prejavom agresivity (Klöppel et al., 2010; Sachdev, n.d.).

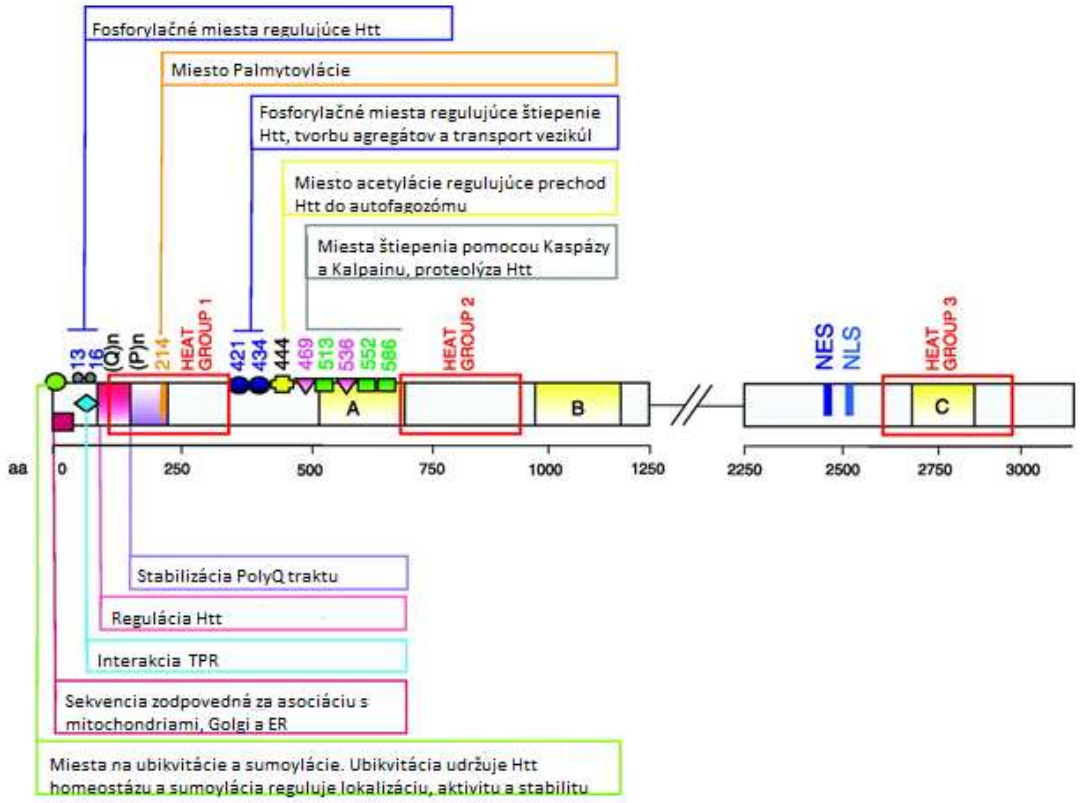
Depresia – ide o častý symptóm, ktorý sa môže prejaviť už niekoľko rokov pred prvými hybnými prejavmi choroby. Časté sú takzvané ranné pessima, kedy pacient o predčasnom prebudení pociťuje intenzívnu úzkosť, má obavy z nového dňa, jeho pocity neschopnosti a zlyhania dosahujú najväčšej intenzity. Časté sú sklony k samovraždám (Jane S. Paulsen, Hoth, Nehl, & Stierman, 2005).

1.2.1. Funkcie a štruktúra proteínu huntingtínu

Má rozhodujúcu úlohu v embryonálnom vývoji, na čo poukazuje fakt, že u myší kompletnou inaktiváciou huntingtínu dochádzalo k odumretiu embrya ešte pred štádiom gastrulácie, a tým pádom to bolo ešte pred zahájením vývoja CNS (Duyao et al., 1995; Nasir et al., 1995). Ak sa zníži huntingtín v neskoršej fáze embryogenézy dochádza k defektnému vývoju mozgu a taktiež je ovplyvnená tvorba CSF (Dietrich, Shanmugasundaram, E, & Dragatsis, 2008; White et al., 1997). S najväčšou pravdepodobnosťou počet CAG repetícií v priebehu vývinu nemá podstatný význam, keďže pacienti s HCH sa vyvíjajú normálne (Leavitt et al., 2001).
Jeho škála pôsobenia je rôznorodá. Pri aktivácii niektorých kaspáz zabraňuje apopotóze (Rigamonti et al., 2000). Taktiež ovplyvňuje vezikulárny transport (Velier et al., 1998). Čo sa týka HCH, je veľmi dôležitý fakt, že podporuje expresiu BDNF (z angl. brain-derived neurotropic factor), ktorý je dôležitý pre prežitie neurónov striata a správne fungovanie nervových synapsií (Gauthier et al., 2004; C. Zuccato et al., 2001). Pokusy na myšiach odhalili, že pokiaľ odstránime nemutovaný HTT, tak mnohé prejavy HCH boli omnoho horšie než u jedincov, ktorý malý endogénny HTT zachovaný. To podporuje hypotézu, že patológiu bunky pri HCH ovplyvňuje aj mutovaný a endogénny HTT (Van Raamsdonk et al., 2005).
Tento proteín sa rozdeľuje na niekoľko oblastí, ktoré hrajú zásadnú úlohu pri fungovaní organizmu. Ako prvá je doména, ktorá interaguje s membránami mitochondrií, s ER a GA. Nazýva sa N- terminálna časť, a skladá sa zo 17aminokyselín (AMK) (Rockabrand et al., 2007). Ďalšiu oblasť nazývame polyQ. Začína od 18 AMK, a vyznačuje sa opakovaním CAG repetícií. Jeho úlohou je interakcia s inými proteínmi. U pacientov dochádza k predĺženiu tohto úseku, čo môže mať za následok abnormálne interakcie (Kim, Chelliah, Kim, Otwinowski, & Bezprozvanny, 2009). U vyšších stavovcov po PolyQ úseku nasleduje polyprolinový úsek PolyP (Elena Cattaneo, Zuccato, & Tartari, 2005). Tento úsek je zodpovedný za rozpustnosť huntingtínu. PolyP asociuje s proteínmi , ktoré regulujú transkripciu. Jeho dĺžka negatívne ovplyvňuje transkripčnú aktivitu proteínu (Elena Cattaneo et al., 2005). Ďalšou doménou, ktorá je v proteíne celkovo 16 krát, a je rozmiestnená v štyroch klastroch je HEAT doména. Jej úloha je umožnenie proteínových interakcií (Elena Cattaneo et al., 2005). Jej dĺžka je približne 40 AMK. Taktiež sa zúčastňuje na prenose molekúl do jadra (E Cattaneo et al., 2001). Na rozvoji patologického stavu majú veľký význam štiepne miesta v proteíne spojené so špecifickými kaspázami (C L Wellington et al., 1998; Cheryl L Wellington et al., 2002). Huntingtín tiež obsahuje aktívny nuclear export signál (NES) sekvenciu a menej aktívny nuclear localization signál (NLS). Tieto sekvencie spôsobujú , že

huntingtín sa účastní na prenose molekúl z jadra do cytoplazmy. Tento názor potvrdzuje i to, že je huntingtín z veľkej časti lokalizovaný nukleárne a perinukleárne, taktiež, že 17 aminokyselín pred polyQ úsekom v sekvencii proteínu interaguje s proteínmi jadrového póru. Keď týchto 17 aminokyselín odstránime, vedie to k hromadeniu huntingtínu v jadre (Cornett et al., 2005).

Huntingtín podlieha mnohým postranslačným úpravám. Fosforylácia, SUMOylácia, ubikvitinácia či acetylácia ovplyvňujú agregáciu a toxicitu proteínu. Pokiaľ bude HTT ubikvitinovaný, tak bude zacielený do proteazómu (Kalchman et al., 1996). Fosforylácia na seríne S421 a S434 chráni HTT pred proteolytickým štiepením., naproti tomu, ak bude fosforylovaný S13 a S 16 prebehne degradácia (Thompson et al., 2009). O bunkovej lokalizácii bude rozhodovať SUMOylácia (Steffan et al., 2004).



Obr.č. 2 ***Domény huntingtínu a miesta postranslačných úprav*** *(upravené podľa (Chiara Zuccato, Valenza, & Cattaneo, 2010)*

1.2.2. Patológia orgánov pri HCH

Už od počiatočných štádií choroby pozorujeme stratu hmotnosti (Djoussé et al., 2002). Tá je zapríčinená viacerými faktormi. K ním zaraďujeme neuroendokrinné zmeny, behaviorálne poruchy, taktiež kognitívne poruchy. V neskorších štádiách dochádza k dysfágii, ktorá následne spôsobí kachexiu. Okrem nedostatočnému príjmu potravy, je u HCH pozorovaný zvýšený metabolizmus. Goodman predpokladá, že zvýšený metabolizmus je zapríčinený dysfunkciou hypotalamu (Goodman et al., 2008). Ako sprievodné javy choroby sú pozorované : osteoporóza, diabetes, rôzne zmeny imunitného systému – hlavne zmeny zápalové (Valekova et al., 2016; van der Burg, Björkqvist, & Brundin, 2009).

1.2.2.1. Patológia mozgu

Pre HCH sú typické nervové príznaky ochorenia. K týmto klinickým črtám dochádza v dôsledku degenerácie v centrálnej nervovej sústave. Halliday vo svojom článku popisuje, že analýzami post mortem dokázal zníženie celkového objemu mozgu o 19 % (v terminálnom štádiu choroby) v porovnaní s kontrolami v rovnakom veku (Halliday et al., 1998). Atrofia je pozorovaná hlavne v nucleus caudatus a putamene, čo sú časti mozgu súhrne nazývanej corpus striatum (Chiara Zuccato et al., 2010). Taktiež sa deteguje významná atrofia v cerebellu pacientov s HCH (Rüb et al., 2013). Prejav nervových príznakov podporuje zníženie hladín kyseliny gama-aminomaslovej (GABA) (Vonsattel & DiFiglia, 1998). Toto zníženie je spôsobené selektívnou stratou stredne dlhých tŕnitých neurónov striata.

1.2.2.2. Patológia kardiovaskulárneho systému

Ako druhá najčastejšia príčina úmrtia pacientov s HCH je zlyhanie srdca. Je uvedené, že až 30 % pacientov s HCH zomrelo z dôvodu zlyhania srdca (Mihm et al., 2007). Mihm taktiež zistil, že u R6/2 myší nastáva zmena výkonu srdca – dochádza k výraznej redukcii systolického a diastolického výkonu srdca a to až tak, že v 12. týždni veku je výkon srdca len 50%. Možnou príčinou môže byť dysregulácia ANS (autonómneho nervového systému). Ten totiž postihuje ako sympatickú , tak aj parasympatickú vetvu (Bär et al., 2008). Pri poruchách ANS dochádza u pacientov s HCH k chaotickému srdečnému rytmu, k vznikom arytmií, ktoré môžu viesť k náhlej arytmickej smrti (Kiriazis et al., 2012).

1.2.2.3. Patológia kostrového svalu

Prejavy patológie HCH môžeme pozorovať aj v kostrových svaloch. Je pozorované svalové plytvanie u pacientov (Turner, Cooper, & Schapira, 2007). Bola dokázaná zvýšená syntéza proteínov v lýtkovom svale u R6/2 myší oproti kontrolám. Pri štúdií, kde sa sledovala svalová sila, bolo u 20 pacientov s HCH pri použití ručného dynamometra zistené, že kontroly boli asi o 50% silnejšie ako pacienti (Busse, Hughes, Wiles, & Rosser, 2008). To môže byť spôsobené svalovou atrofiou. Tá postihuje pomalé oxidačné červené vlákna s vysokým obsahom myoglobínu, ale aj rýchle glykolytické vlákna so strednou a nízkou oxidačnou kapacitou (Gizatullina et al., 2006; Ribchester et al., 2004). Iné práce poukazujú na apoptózu v svalových bunkách, na mitochondriálnu depolarizáciu, na zvýšené uvoľňovanie cytochrómu c do cytosolu. Taktiež je dôležité zvýšenie aktivity kaspázy 3, kaspázy 8 a kaspázy 9 (Ciammola et al., 2006).

1.2.2.4. Testikulárna patológia

Najväčšia expresia proteínu Htt prebieha v mozgu a semenníkoch (S. H. Li et al., 1993). Rozsah expresie v semenníkoch je porovnateľný s expresiou v mozgu (Guo et al., 2003). Na myších modeloch HCH je pozorovaná atrofia semenníkov, poškodenie semenotvorného epitelu a znížený počet zárodočných buniek (Papalexi et al., 2005; Van Raamsdonk et al., 2005, 2007). Pacienti s HCH, u ktorých prebehli vyšetrenia semenníkov, majú detegované testikulárne abnormality, redukciu počtu zárodočných buniek a abnormálnu morfológiu semenotvorných kanálikov (Van Raamsdonk et al., 2007). Niektorí autori sa zaoberali otázkou, že či poškodenie testes je spôsobené len prítomnosťou mtHtt, alebo či fenotyp prejavujúci sa testikulárnou degeneráciou môže ovplyvniť i poškodenie neurónov, ktoré sú zodpovedné za hormonálne zmeny, a tým sa poruší hormonálna os hyptalamus – hypofýza – gonády. Markianos vo svojej štúdii taktiež zistil zníženú koncentráciu hormónu testosterón (Markianos, Panas, Kalfakis, & Vassilopoulos, 2005). Naproti tomu štúdie Saleha nepreukázali signifikantné zmeny v koncentrácii testosterónu medzi pacientami s HCH a zdravými kontrolami (Saleh et al., 2009).

1.2.2.5. Správanie a zmeny správania pri Huntingtonovej chorobe

Správanie je odpoveď organizmu (alebo systému) na rôzne podnety, či už vnútorné alebo vonkajšie, pri vedomí alebo podvedomí. Tieto podnety môžu byť zjavné alebo skryté (Minton

& Kahle, n.d.). Vo vedeckej literatúre sa uvádza, že správanie je vnútorne koordinovaná reakcia (činnosť alebo nečinnosť) živých organizmov na vnútorné a vonkajšie podnety (Levitis, Lidicker, Freund, & Freund, 2009). Širšia definícia správania organizmov je podobná konceptu fenotypovej plasticity. Popisuje, že správanie (reakcia na udalosť) sa mení v priebehu celého života jedinca (Karban, 2008). Dá sa povedať, že je to odpoveď organizmu na dianie okolo neho (Dusenbery, 2009). Táto odpoveď môže byť vrodená alebo naučená. U ľudského správania sa očakáva, že bude ovplyvnené endokrinným a nervovým systémom. Predpokladá sa, že zložitosť správania organizmu koreluje zložitosti jeho nervovej sústavy. Všeobecne platí, že organizmy so zložitou nervovou sústavou majú väčšiu schopnosť učiť sa nové odpovede a tým prispôsobiť svoje správanie (Dowhan , 2013).

Zmeny správania pri Huntingtonovej chorobe

Správanie je zaujímavý a dôležitý parameter v mnohých disciplínach zaoberajúcimi sa neurodegeneráciou. Štúdium správania je dôležité na určenie welfare zvierat, zmien správania pri neurogeneratívnych ochoreniach a taktiež pri poškodeniach mozgu (Lind, Vinther, Hemmingsen, & Hansen, 2005). Pacienti s HCH majú veľké problémy s koordináciou a schopnosťou učiť sa. Okrem mimovoľných pohybov končatín a zhoršenej koordinácie, majú zhoršenú výslovnosť. Taktiež schopnosť učiť sa s postupom ochorenia klesá.

1.3. Terapia Huntingtonovej choroby

V dnešnej dobe zatiaľ nie je k dispozícií žiadna liečba, ktorá by spomalila a zastavila progresiu ochorenia. Používajú sa len farmaká na dočasné zmiernenie klinických príznakov (Ross & Tabrizi, 2011). Pri progresii ochorenia sa začne prejavovať úbytok na váhe. Pacient s HCH s prejavmi chudnutia potrebuje približne dvojnásobok kalorického prímu zdravej osoby, čo predstavuje približne 5000 kcal na deň (Roth et al., 2009). Pri dysfágii je dôležité pripravovať jedlo na malé kúsky, dbať na správnu hustotu – najlepšie kašovitá (Roth et al., 2009). Na zmiernenie chorei sa používajú neuroleptiká. Najčastejšie podávaný je tetrabenazin na zredukovanie mimovoľných pohybov. Podávanie liečiv, ako sú antioxidanty, protiapoptotické ATB a antagonisti glutamátu, určené na zmiernenie motorických a kongnitívnych prejavov HCH , dosiahli len obmedzenú účinnosť (Ramos et al., 2013). Netreba zabúdať, že pri liečení pacientov bez zjavných symptómov je potrebné poznať

indikátory ochorenia, tzv. biomarkery. Okrem toho, že nám dovoľujú sledovať progresiu ochorenia, taktiež nám môžu poskytnúť dôležité poznatky, či poskytnutá liečba je úspešná.

1.3.1. Terapia Huntingtonovej choroby pomocou génovej terapie

Génovou terapiou nazývame liečebný postup, pri ktorom je do genómu pacienta vložená sekvencia DNA, pričom táto sekvencia kóduje chýbajúci alebo nefungujúci proteín, poprípade môže potlačiť mutovaný proteín. Podľa toho či terapia nahradí gény vo všetkých telových bunkách okrem pohlavných, alebo nahradí gény v gamétach delíme terapiu na somatickú a gametickú. V génovej terapii Huntingtonovej choroby sa najčastejšie využívajú ASO (antisense oligonukleotidy), mRNA interferencia, zink-fingers a CRISPR-CAS9 metódy. Ich úlohou je zníženie transkripcie mutovanej DNA. Zníženie HTT pomocou ASO bolo úspešné na zvieracích modeloch (Keiser, Kordasiewicz, & McBride, 2016). V septembri 2015 bola prvýkrát podaná pacientom terapia IONIS-HTTRx založená na princípe ASO (Wild & Tabrizi, 2017). Experimenty na myšiach dokázali zníženie expresie HTT a expresie proteínu asi o 2 tretiny (Bennett & Swayze, 2010). Taktiež na zvieracích modeloch HCH boli úspešné prístupy na princípe siRNA (Keiser et al., 2016). Stiles použil infúzne podanie siRNA bez vírusového vektora od putamenu primátov. Dosiahol supresiu HTT na celom striate (Stiles et al., 2012). Terapie zamerané na DNA zahrňujú zink-fingers a CRISPR-CAS9. Obe metódy dosahujú úspechy. CRISPR-CAS9 bola v r. 2017 úspešne testovaná na myšaciom modely HCH. Došlo k zmierneniu patológie, zlepšeniu motorických funkcií a predĺženiu prežitia (Yang et al., 2017)

Génová terapia Huntingtonovej choroby			
	Alelová selektivita	Vector	Sponzor
ASO			
Pre-mRNA degradation	None	None	Ionis Pharmaceuticals
Pre-mRNA degradation	SNP-targeted	None	Wave Life Sciences
Pre-mRNA degradation	CAG repeat	None	Biomarin
RNA interference compounds			
mRNA degradation	None	AAV2	Spark
mRNA degradation	None	AAV1	Voyager
mRNA degradation	None	AAV5	Uniqure
Small molecules			
Screening progamme	unknown	None	CHDI Foundation

Obr.č. 3 ***Štúdie génovej terapie,*** *Ionis – klinická, zvyšné sú v predklinickej fáze (upravené podľa (Wild & Tabrizi, 2017))*

1.4. Zvieracie modely Huntingtonovej choroby

Na vyvinutie správnej liečby pre ľudí trpiacich HCH je potrebné porozumieť nielen prejavom choroby, ale aj mechanizmom, ktoré túto chorobu spôsobili. Aby sme tieto mechanizmy mohli študovať, je potrebné vytvoriť zvieracie modely, ktoré budú mať fenotyp odpovedajúci HCH. V dnešnej dobe existuje niekoľko modelov, a každý z nich má svoje výhody i nevýhody. Ako výhodný zvierací model by sa mohol javiť primát, pretože svojou veľkosťou a fyziológiou je najviac podobný človeku (Chan & Agca, 2008), lenže z etického hľadiska je to v mnohých štátoch zakázané, jeho cena je vysoká a nedostatok potomkov nedovoľuje podrobné preštudovanie patológie choroby.

Medzi prvé zvieracie modely HCH patrili modely bezstavovcov. Ich nesmiernou výhodou je rýchle testovanie hypotéz a terapeutických stratégií. Táto výhoda je spôsobená produkciou veľkého počtu potomkov. Najčastejšie požívaná je *Drosophila melanogaster*, exprimujúca dlhé polyglutamínové repetície v oku a *Caenorhabditis elegans*, ktorý exprimuje dlhý polyglutamínový reťazec v nervovom systéme (Ramaswamy, McBride, & Kordower, 2007). Prechod medzi bezstavovcami a stavovcami tvorí zástupca nižších stavovcov ryba *Danio rerio*. Veľkou výhodou tejto rybky je, že má kompletne oskenovaný genóm a dobrú dostupnosť rôznych mutantov. Jej embryonálny vývoj je veľmi rýchly , embrya sú veľké a priehľadné, vyvíjajúce sa mimo tela matky.

Najviac podobné človeku sú však zvieracie modely cicavcov. Cicavčie modely môžeme rozdeliť na genetické a negenetické. Ako prvé sa rozvíjali negenetické modely. Tie sú založené na indukcii bunečnej smrti vplyvom excitotoxicity. Na jej vyvolanie sa najčastejšie používali kyselina chinolinová a kyselina kainová (Schwarcz, Foster, French, Whetsell, & Köhler, 1984). Ako mitochondriálny toxín sa používa 3-nitropropionová kyselina. Genetické modely môžeme rozdeliť na modely s náhodne vloženým transgénom, ktoré exprimujú skrátený alebo plne dlhý mutantný huntingtín a na knock-in modely (Ramaswamy et al., 2007). Najviac využívané sú myši. Ale taktiež sú vyvinuté modely na potkanoch, prasatách ovciach a opiciach. Aj keď je nepopierateľné, že hlodavce poskytli dôležité informácie o patofyziológii HCH, ich využitie je limitované. V porovnaní s človekom majú veľmi malý mozog a inú anatómiu mozgu. Keďže ich dĺžka života je krátka, tým pádom nie je možné urobiť dlhodobú štúdiu. Aby sa mohli robiť dlhodobé štúdie, boli vytvorené modely veľkých zvierat. Tie zahrňujú opice, prasatá a ovce (Morton & Howland, 2013). Veľké zvieracie modely majú niekoľko výhod. Najdôležitejšia je podobná štruktúra mozgu s človekom.

Výhodou TgHD miniprasiat je váha v dospelosti 70 -90kg, čo je podobné ako u človeka, veľký gyrencefalický mozog s podobnou neuroanatómiou ako majú ľudia .Taktiež môžeme sledovať progresiu ochorenia pomocou zobrazovacích techník ako je MRI a PET (Bjarkam, Jorgensen, Jensen, Sunde, & Sørensen, 2008; X.-J. Li & Li, 2012).

1.4.1. Liběchovský model transgénneho miniprasaťa pre HCH

Na akadémii vied ČR v Liběchove, pod vedením prof. Jana Motlíka z Ústavu živočíšne fyziológie a genetiky v roku 2009 sa tímu vedcov podarilo vytvoriť model transgénneho miniprasaťa pre Huntingtonovu chorobu. Tento model nesie časť sekvencie kodújúcu ľudský mutovaný huntingtín (Baxa et al., 2013). Tento transgénny model bol vytvorený mikroinjekciou lentivírusového vektoru, ktorý obsahoval promotorovú sekvenciu pre ľudský huntingtin a N-terminálnu skvenciu (548 AMK) s 145CAG/CAA repetíciami do embryí miniprasiat v štádiu prvojadier. Insert je lokalizovaný na chromozóme 1. V máji 2009 sa narodila prasnička Adélka, nesúca mtHtt, ktorá sa následne stala zakladateľkou línie transgénnych miniprasiat. Exprimuje N-terminálny fragment mutovaného huntingtínu so 124 CAG/CAA repetíciami. Výhodou tohto modelu je väčší počet potomkov na jeden vrh, a pri dĺžke gravidity necelé 4 mesiace môže prasnica porodiť 2 krát v roku. Podiel TG a WT potomkov je približne 1:1 (Baxa et al., 2013; Kotrcova et al., 2015).

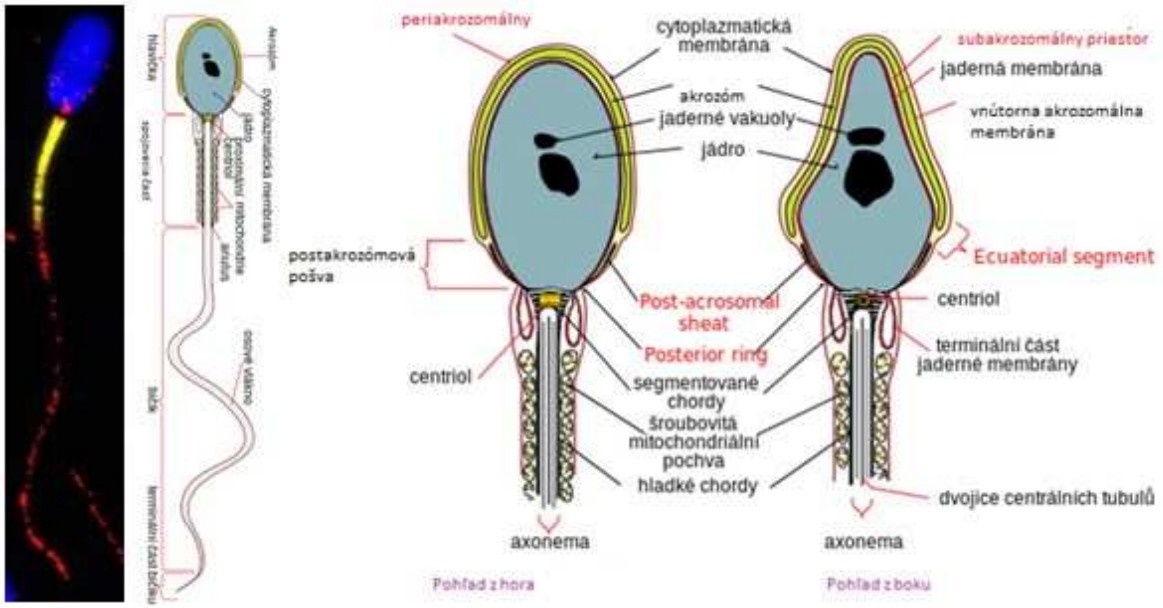
Obr.č. 4 ***Prvý vrh prasiat vzniknutých z injektovaných embryí, medzi prasiatkami TG prasnička Adélka*** *(zdroj Centrum PIGMOD)*

1.5. Zloženie ejakulátu kancov

Ejakulát je biologická tekutina ktorá obsahuje 93-97% semennej plazmy a 3-7% spermií (Kudláč, 2003; Říha et al., 2003). Zloženie semennej plazmy je približne 3 % sekrétu prisemenníkov, 20% sekrétu semenných váčkov, 15 % sekrétu bulbouretrálnych žliaz a 62 % sekrétu prostaty (Říha et al., 2003). Vylučovanie spermií kancov môžeme rozdeliť do niekoľkých fáz. Tieto fázy nazývame frakcie. Prvá je prespermiová frakcia. Po nej nasleduje spermiová frakcia a nakoniec postspermiová. Najviac spermií sa vylúči v spermiovej frakcii, je to 80-90% všetkých spermií počas ejakulácie (Čeřovský, 2001; (Saravia et al., 2009)).

1.5.1. Stavba a funkcia spermií

Spermie sú špecializované bunky s typickou štruktúrou. Ich úlohou je predanie genetického materiálu obsiahnutého v jadre spermie do cytoplazmy oocytu (Eddy, 2006). Svojou morfologickou stavbou predstavujú bunku s cielenou funkciou – oplodnenie. Celková dĺžka spermie tvorená hlavičkou a bičíkom sa pohybuje okolo 60µm (Říha et al.,2003). Základ hlavičky tvorí vysoko kondenzované jadro. Na povrchu hlavičky je akrozóm, ktorý je naplnený enzýnmi. Tieto enzýmy sú potrebné na rozpustenie obalov okolo oocytu a tým sa uľahčí penetrácia spermie do jeho vnútra (Říha et al.,2003, Eddy, 2006). U kanca akrozóm pokrýva 70% dlhej osy hlavičky (Vežník et al., 2004), a je veľmi labilný. Pri patologických procesoch v pohlavnom ústrojenstve a taktiež pri náhlych zmenách osmotického tlaku, pri zmenách teplôt prostredia, pri rozdielnom pH dochádza veľmi ľahko k jeho poškodeniu (Kliment et al., 1989). Bičík je zložený z centriolového oddielu – tzv. krček spermie, mitochondriálneho aparátu, axiálnych vlákien tvoriacich spojovaciu časť a hlavného oddielu zakončeného terminálnou časťou bičíka (Gamčík et al., 1976). Tvorbu energie zabezpečuje spojovacia časť bičíku a premenou na energiu mechanickú umožňuje spermii pohyb (Říha et al.,2003, Eddy, 2006). Cytoplazmatická membrána, ktorá pokrýva celý povrch hlavičky aj bičíka zaisťuje ochranu spermií pred nepriaznivými podmienkami. Je acidorezistentná s vysokou schopnosťou permeability. Zabezpečuje látkovú výmenu medzi spermiou a prostredím (Marvan et al., 1992). Kančie spermie majú vysokú koncentráciu nenasýtených mastných kyselín v plazmatickej membráne a nedostatočný enzymatický antioxidačný systém. To spôsobuje, že sú náchylné k nadmernému pôsobeniu reaktívnych foriem kyslíku (Cerolini, Maldjian, Surai, & Noble, 2000; Strzeżek et al., 2005).



Obr.č. 5 ***Stavba spermie*** (upravené z *"Gray's anatomy" 36th edition, Williams & Warwick, 1980 a Krizova et al., 2017)*

1.5.2. Spermiogenéza

Spermiogenéza začína v období pohlavného dospievania. U samcov je to počas celého obdobia pohlavného života (Bohuslavová, Mačáková, 2015). Prebieha v semenotvorných kanálikoch. Nástup je riadený pomocou gonadotropných hormónov hypofýzy. Spermiogenéza je regulovaná pomocou folikulo-stimulačého hormónu (FSH) a skladá sa z troch fáz – rozmnožovacej, rastovej a zrecej (O'Donnell, Nicholls, O'Bryan, McLachlan, & Stanton, 2011). Najskôr cicavčie zárodočné primordiálne bunky migrujú na pohlavnú ryhu samčieho embrya, kde sa začlenia do pohlavnej línie. Tam zostávajú v kľude až do pohlavnej dospelosti. V období dospievania sa diferencujú somatické Sertoliho bunky. Tie následne zaistia a umožnia spermiogenézu, pretože chránia a vyživujú vyvíjajúcu sa spermatickú bunku. Pretože Sertoliho bunka sa už v období pohlavnej dospelosti nedelí, a jej obnova nie je možná , je tým pádom limitujúcim faktorom spermiogenézy.

Rozmnožovacia fáza

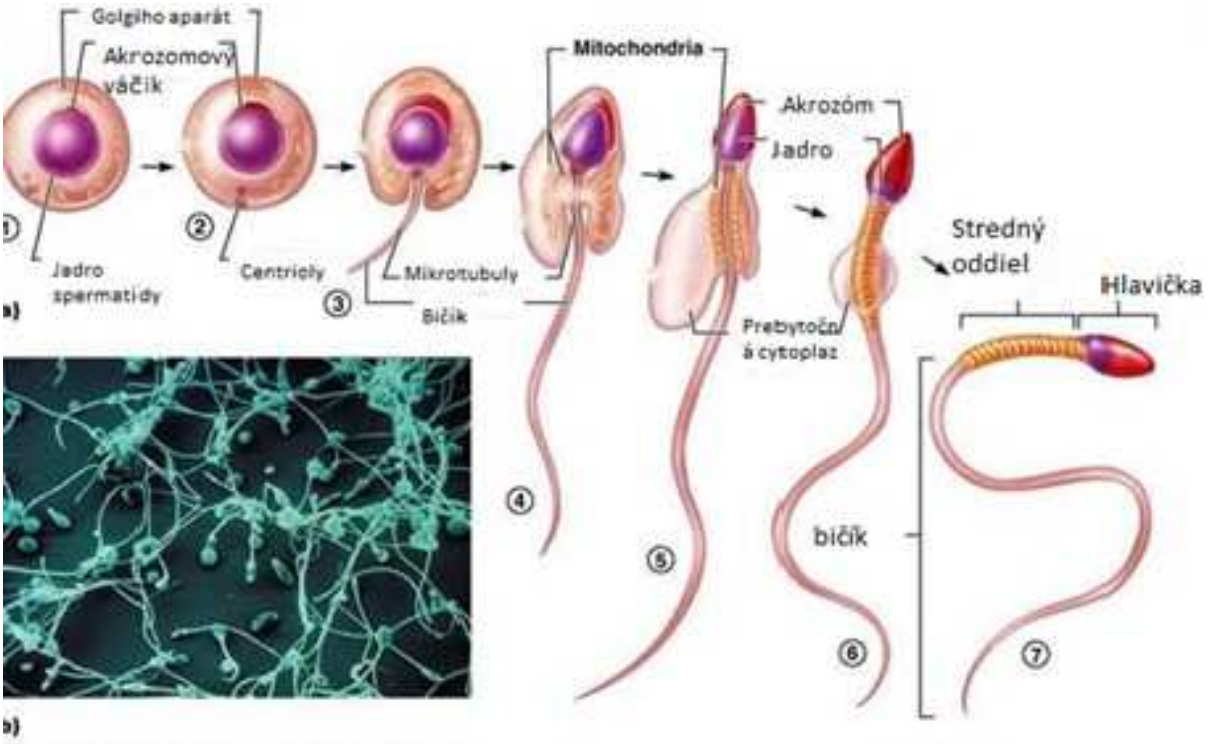
Keď prvotné zárodočné germinálne bunky vstúpia do seminiferného epitelu semenníkov tak sa začnú deliť do formy spermatogónie 1.rádu –A1. Pre ne je charakteristické vajcovité jádro obsahujúce jadrový chromatín pridružený k jadrovej membráne. Po dosiahnutí pohlavnej dospelosti sa začínajú deliť na spermatogónie A2. Tie sa môžu ďalej deliť na ďalšie spermatogónie, môžu podstúpiť apoptózu, alebo sa môžu diferencovať na intermediálne spermatogónie.

Rastová fáza

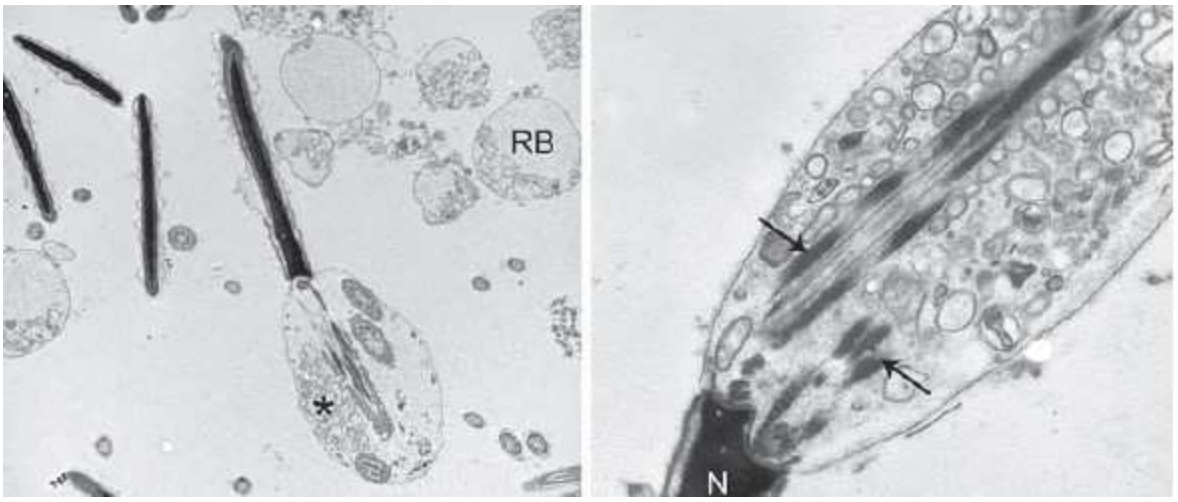
Intermediálna spermatogónia sa mitoticky delí do formy spermatogónie typu B – spermatogónia 2.rádu. Tá je prekurzorom spermiocytu. Spermiocyt vstupuje do meiózy. GDNF (Glial cell-Derived Neurotrophic Factor – neurotrofický faktor pochádzajúci z gliálnejí línie), je sekretovaný Sertoliho bunkami a sprostredkováva prechod medzi spermatogóniami a spermiocytmi. Jeho hladina určuje, či deliaca sa spermatogónia zostanie spermatogóniou alebo sa bude diferencovať na spermiocyt. Nízka hladina GDNF – diferenciácia, vysoká – zotrvanie vo forme spermatogónie (Meng et al., 2000). Vytvorené haploidné bunky po druhom meiotickom delení sa nazývajú spermatídy. Tie zostávajú navzájom spojené pomocou cytoplazmatických mostíkov a tvoria tzv. syncítium. Počas delenia z typu A1 spermatogónie na spermatídu sa pohlavné bunky pohybujú ďalej od základnej membrány semenotvorných kanálikov smerom do stredu lúmenu.

Fáza zrenia – diferenciačná (spermiohistogenéza)

Haploidná spermatída cicavcov je guľatá, bezbičikatá bunka. Svojím vzhľadom nepripomína dospelú spermiu. Konečnú podobu získava počas spermiohistogenézy. Ako prvý sa vytvára akrozomálny váčik. Ten vzniká zväčšením Golgiho aparátu. Akrozóm pokrýva jadro spermie. Vo váčku má prítomné enzymatické proteíny ako fosfatáza, hyaluronidáza, neuraminidáza (Flörke, Phi-van, Müller-Esterl, Scheuber, & Engel, 1983). Po vytvorení akrozomálneho váčika sa jadro pretočí. Táto rotácia je potrebná pre tvorbu bičíka. Ten sa začne tvoriť z centrioly na druhej strane od jadra, a bude rásť smerom do lúmenu kanálika. Počas poslednej fázy spermiohistogenézy sa jadro splošťuje a kondenzuje. Zvyšná cytoplazma sa odlúči v podobe cytoplazmatickej kvapky.



Obr.č. 6 ***Spermihistogenéza*** *(*upravené z *Copyright © 2004 Pearson Education, Inc., publishing as Benjamin Cummings Fundamentals of Anatomy & Physiology SIXTH EDITION Frederic H.)*



Obr.č. 7 ***Spermie s cytoplazmatickou kvapkou*** *(z Macakova, Bohuslavova et al., 2016)*

1.6. Patológia ejakulátu

Oligospermia

Oligospermia je znížená tvorba spermií. Je najbežnejšou príčinou neplodnosti samcov. Faktory, ktoré spôsobujú oligospermiu môžu byť vrodené a získané (Rowe & World Health Organization., 2000). K vrodeným faktorom zaraďujeme endokrinné a urologické patológie. Najčastejšie urologické poruchy sú varikokéla (Costabile & Spevak, 2001) – rozšírenie ciev v oblasti mieška, a chronické infekcie močových ciest. K endokrinným poruchám spôsobujúcim oligospermiu zaraďujeme nedostatok gonadotropínu, vysokú hladinu kortizolu spôsobenú Cushingovým syndrómom, hyperprolaktémiu, hemochromatózu pri ktorej dochádza k nadmernému vstrebávaniu železa z tráviaceho traktu a jeho následnému ukladaniu v orgánoch a celkovú nedostatočnosť hypofýzy. K získaným faktorom zaradujeme prehrievanie organizmu, nedostatočnú výživu hlavne deficit vitamínu C, selénu, zinku a kyseliny listovej, obezitu, otravy ťažkými kovmi (Menzies, Shepherd, Nibbs, & Nelson, 2011).

Azospermia – neprítomnosť spermií v ejakuláte

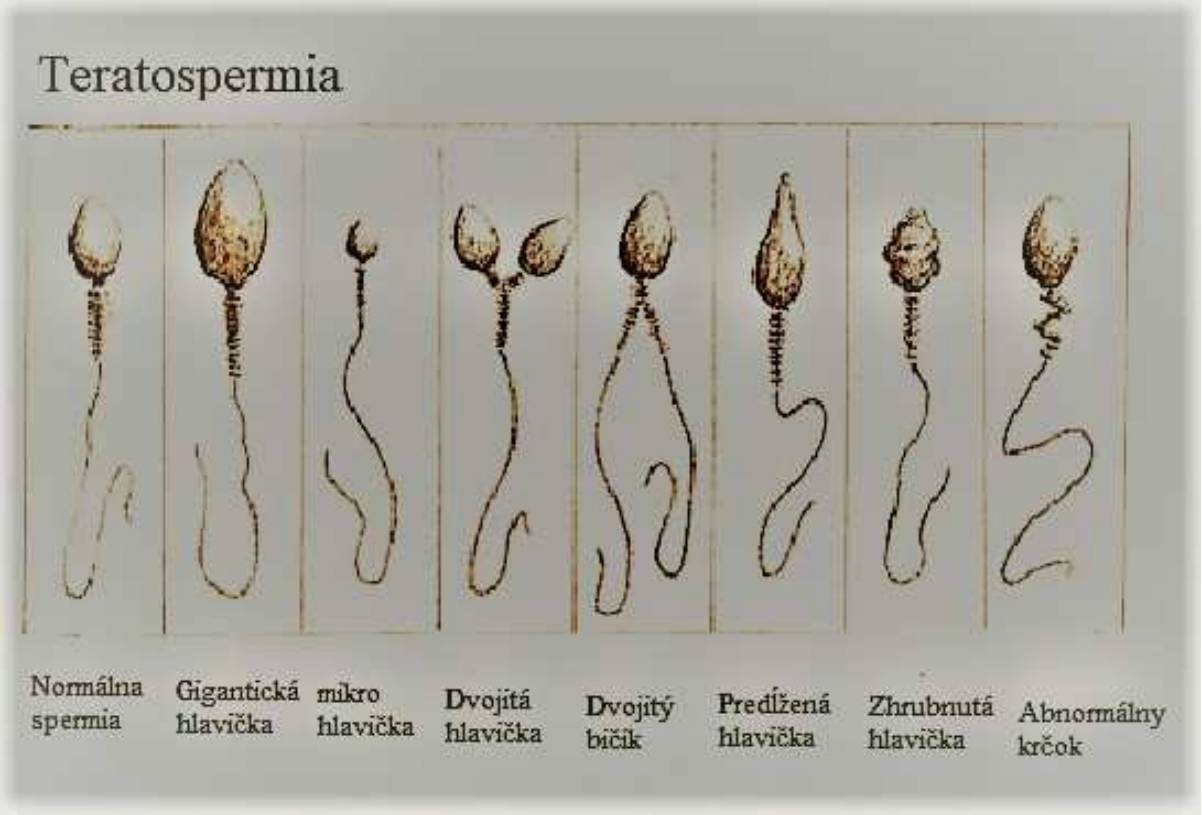
Presná definícia azospermie je neprítomnosť zrelých spermií v centrifugovanom vzorku ejakulátu (Zvěřina, 2003). Azospermiu delíme na obštrukčnú a neobštrukčnú. Obštrukčnú azospermiu spôsobuje prerušenie komunikácie medzi semenníkom a močovou trubicou, čím sa zabráni vstupu spermií do ejakulátu. Neobštrukčná azospremia je spôsobená výrazne zníženou produkciou spermií. Jej príčinou môže byť agenéza (nevyvinutie orgánu), alebo zastavenie dozrievania spermií (Kolárová, Vrtěl, Vodicka, Capková, & Santavý, 2003)

Nepohyblivosť spermií – asthenospermia

Ak máme v odobratej vzorke zníženú pohyblivosť menej než 40% pohyblivých spermií hovoríme o astenospermii (WHO, 2010). Často sa pridružujú morfologické abnormality bičíku ako nesprávna konštrukcia axonémy (Mitchell et al., 2006; Moretti, Pascarelli, Federico, Renieri, & Collodel, 2008) a fibróznej pošvy (Escalier, 2003).

Patologická morfologi spermií – teratospermia

Ak máme v ejakuláte zníženú hladinu spermií normálnej morfológie na 15% a menej hovoríme o teratospermii (Machev, Gosset, & Viville, 2005). Rozlišujeme ľahkú teratospermiu – 10 – 15 % spermií ma normálnu morfológiu a ťažkú teratospermiu - menej než 4% spermií s normálnou morfológiou (WHO, 2010). Často príčinou je genetický faktor. Veľmi vzácny prípad teratospermie je globospermia (spermia s guľatou hlavičkou). U takejto spermie väčšinou chýbajú akrozomálne štruktúry, a tý pádom ma spermia zníženú schopnosť viazať sa k zone pellucide a preniknúť do oocytu (Edirisinghe, Murch, Junk, & Yovich, 1998; Rybouchkin, Dozortsev, Pelinck, De Sutter, & Dhont, 1996; Vicari et al., 2002).



Obr.č. 8 ***Teratospermia*** *(upravené z http://wombwithastory.blogspot.cz/2010/02/sperm-morphology-teratozoospermia.html)*

2. CIELE

Cieľom dizererтačnej práce bolo popísať fenotyp transgénneho miniprasaťa s HCH.

1. Prvým cieľom dizertačnej práce bolo popísať funkčné a štrukturálne zmeny spermií kancov miniprasiat transgénnych pre HCH a ich kontrólnych súrodencov.

2. Druhým cieľom dizertačnej práce bolo štúdium metabolických zmien v semenníkoch u F2 generácie kancov miniprasiat transgénnych pre HCH použitím ^{31}P MRI spektroskopie.

3. Tretím cieľom bolo štúdium mitochondriálnej dysfunkcie spermií kancov miniprasiat transgénnych pre HCH.

3. METÓDY

3.1. Experimentální model

Použitý model: transgénne miniprasa pre Huntingtonovu chorobu

Ústav živočíšne fyziológie a genetiky AVČR v Liběchově dlhodobo chová a rozmnožuje nepríbuzenskou plemenitbou miniatúrne prasatá, ktoré sú potomkami importovaných miniprasiat z University v Gottingen a University v Minesote a ich potomkov. Netransgenné prasničky vo veku 6 až 8 mesiacov boli ako darkyne jednobunkových embryí, tak príjemcami embryí po mikroinjekci lentivírusového vektoru do perivitelinného priestoru. Podarilo sa tak vytvoriť v roku 2009 jedinečný, veľký zvierací model Huntingtonovej choroby (Baxa et al., 2013), Naše miniprasa nesie N-terminálny fragment ľudského mutovaného huntingtínu so 124 CAG/CAA repetíciami

Všetky experimenty boli vykonané so súladom ustanovenia pre zachovanie welfare zvierat so súhlasom Štátnej veterinárnej správy Českej republiky.

3.2. Odber semena a testy na určenie parametrov semena

Odber semena – spermiovej frakcie

Odber semena bol vykonávaný ručne. Kance sa najprv museli naučiť skákať na fantóm. Po vložení fantómu do koterca ku kancovi, kanec vyskočil na fantóm, a po začatí páriacich sa pohybov mu bol chytený penis a odobraná spermiová frakcia ejakulátu. Tá sa následne uložila do termosky, ktorá bola vyhriata na telovú teplotu.

Stanovenie počtu spermií, progresivity a motility

V laboratóriu sa odobratý ejakulát prefiltroval, aby sme sa zbavili prípadných nečistôt z odberu. Po filtrácii sa zaznamenal objem odobraného ejakulátu a následne boli zmerané

hodnoty progresivity , motility a počty spermií pomocou SCA analyzátora (Microptic, Spain), (semeno sa stále udržiavalo v teple, pri hodnotách podobných telesnej teplote).

Motilita- celkový pohyb spermií

Progresivita- pohyb spermie za hlavičkou

Obr.č. 9 ***Sperm cell analyzer*** *(Microptic, Spain)*

Test prežívateľnosti spermií (zdroj Centrum PIGMOD)

Najprv sme museli spermie spracovať. K spracovaniu sme používali BTS riedidlo (obr.č.10). K ejakulátu sme pridali BTS riedidlo a centrifugovali pri 600G 20 min a teplote 24 ºC. Potom sme odstránili supernatant, spermie sme preniesli do 15 ml skúmavky a doliali BTS doplna. Centrifugovali sme ďalších 10 minút pri 600G. Po odstránení supernatantu sme spermie, čo zostali na dne skúmavky rozuspendovali v pomere 1:1 s BTS riedidlom. Skúmavky so vzorkami sme dali do polystyrénovej krabičky, v ktorej bola teplá voda, a užili do chladničky (kvôli postupnému schladeniu spermií, náhly pokles teploty by ich zničil). V priebehu 5 dní za sebou sme zo vzoriek každý deň merali hodnoty progresivity a motility, po zahriatí vzorky na 36 ºC a zriedení vzorky 1:50 s BTS riedidlom.

Penetračný test

Získavanie oocytov

5 prasničiek bolo počas 15 dní synchronizovaných pomocou Regumate (Jenssen Pharmaceutical) na vyvolanie Estru. Superovulácia bola vyvolaná injekciou PMSG (pregnant mare's serum gonadotropin) (Foligon, Intervet International B.V.) na 15 deň kŕmenia Regumetom. Po 48 hodinách od napichania PMSG sa prasnice humánne zabili, a vyizolovali sa oocyty.

1.deň

Vopred pripravený kultivačný roztok na oocyty a manipulačný roztok (obr.č.10) na oocyty sme si nechali zahriať v inkubátore (inkubátor bol nastavený na 37 ºC, 7% kyslíka). Izolácia oocytov prebiehala o 13 hodine. Oocyty sme dávali do vopred zohriateho manipulačného média. Po dokončení izolácie sme ich z manipulačného média preniesli do teplého kultivačného média (obr.č.10) a nechali inkubovať v termostate do nasledujúceho dňa (inkubátor bol nastavený na 37 ºC, 5% CO_2, 7% kyslíka).

2.deň

O 9 hodine ráno sme prenášali inkubované oocyty do nového manipulačného média a nechali inkubovať do druhého dňa (inkubátor bol nastavený na 37 ºC, ,5% CO_2, 7% O_2).

Taktiež sa odobrali spermie, spracovali pomocou BTS riedidla. Potom sme do skúmavky dali 400µl žĺtok-laktózy a na to sme navrstvili 400µl vzorky semena. To sme potom uložili do polystyrénovej chladiacej veže do druhého dňa (ta udržovala teplotu okolo 17ºC)

3.deň

Ráno o 7 hodine boli oocytom odstránené kumulárne bunky za pomoci Hyásy (Sigma

Aldrich). Oocyty boli následne prenesené do oplodňovacieho roztoku (obr.č.10) vyhriateho na 37 ºC. Na inkubátore sme znížili prívod kyslíka zo 7% na 5%. Kým sme si pripravili spermie na oplodňovanie, oocyty sme nechali inkubovať v termostate.

Príprava spermií na oplodnenie

Vzorky semena sme vybrali z chladiacej veže. Do 2,5 ml manipulačného média pre spermie (obr.č.10) sme pridali 50µl semena. Po premiešaní sme 2 minúty centrifugovali pri 200G a 26ºC. Následne sme supernatant zľahka preniesli do druhej skúmavky a centrifugovali 8 min pri 250G. Supernatant bol použitý na oplodnenie. Množstvo koľko supernatanu sa použije k oplodneniu sme si vopred vyrátali aby koncentrácia spermií bola 1 milión na ml. Po pridaní spermií k oocytom sme ich nechali 15 minúť inkubovať. Následne sme preniesli oocyty i s naviazanými spermiami do čistého oplodňovacieho roztoku a nechali inkubovať do ďalšieho dňa.

4.deň

Po vybratí oocytov z termostatu sa robili vzorky, ktoré by sa dali pozorovať pod mikroskopom. Na podložné sklíčko sme ukladali oocyty približne do rady v počte 10 kusov. Po prikrytí krycím sklíčkom sme ich nechali fixovať do nasledujúceho dňa vo fixačnom roztoku (10ml kyseliny octovej + 30 ml etanolu).

5.deň

Vzorky sme nafarbili orceínom pre lepšiu viditeľnosť oocytov a preniesli s fixačného roztoku do diferenciačného média (15ml kyselina octová + 15 ml etanol+ 15 ml destilovanej vody). Nasledujúce dni sme vzorky pozorovali pod mikroskopom a vyhodnocovali percento úspešnosti oplodnenia.

Počet spermií, progresivita, motilita a penetračné testy boli štatisticky vyhodotené pomocou softvéru GraphPad Prism 5,0. Na vyhodnotenie sa použil Kolmogorov-Smirnov test normality a na štatistické vyhodnotenie Mann.Whitney U test.

Zloženie roztokov používaných pri penetračných testoch a BTS riedidla

Kultivačný roztok oocyty	Manipulačný roztok oocyty	Manipulačný rotok spermie	Oplodňovací roztok spermie	BTS riedidlo
50 ml	50 ml	50 ml	50 ml	250 ml
199H (TCM 199) 8,4ml	199H (TCM 199) 9,2ml	NaCl 330mg	NaCl 330mg	Glukóza 9,2g
7,5% NaHCO3 3,9 ml	7,5% NaHCO3 1,0 ml	KCl 15mg	KCl 15mg	Citrát sódny 1,5g
Ca-Laktát 50mg	Ca-Laktát -	Na-Laktát 50mg	Na-Laktát 50mg	EDTAx2H_2O 0,31025g
Na-Pyruvát 20mg	Na-Pyruvát 20mg	Ca-Laktát 100mg	Ca-Laktát 100mg	NaCl 0,0312g
PVA -	PVA 300mg	KH_2PO_4 5mg	KH_2PO_4 5mg	KCl 0,02g
Hepes acid 200mg	Hepes acid 200mg	$MgCl_2x6H_2O$ 10mg	$MgCl_2x6H_2O$ 10mg	7,5% NaHCO3 4,2ml
Glutamín 10mg	Glutamín 10mg	Na-Pyruvát 8mg	Na-Pyruvát 8mg	AmfotericínB 125µl
Streptomicín 7mg	Streptomicín 7mg	Hepes Acid 235mg	Hepes Acid 35mg	Kanamycín 0,02g
AmfotericínB 100µl	AmfotericínB 100µl	Coffein 30mg	Coffein -	
Kanamycín 8mg	Kanamycín 8mg	PVA 105mg	PVA 105mg	
		Kanamycín 8mg	Kanamycín 8mg	
		Streptomycín 3,5mg	Streptomycín 3,5mg	

Obr.č. 10 ***Zloženie roztokov používaných pri penetračných testoch***

Imunocytochemické pokusy

Spermie boli pomocou cytospinu (800G,5min) nanesené na čisté podložné sklíčko. Pri pokuse boli fixované metanolom 5 minút a následne acetónom na 30s. Sklíčka boli blokované 5 % kozím sérom a 5% mliekom na 30 minút. Následne boli inkubované 2 hodiny s protilátkou 3B5H10 (3B5H10, Sigma Aldrich) v riedení 1:500 pri 4°C. Ako sekundárna protilátka sa použila Alex Fluor 488 (A21424,Invitrogen) v riedení 1:500 na 1

hodinu. DAPI bolo pridané v montovacom médiu. Sklíčka boli pozorované pomocou skenovacieho mikroskopu Olympus BX.

3.3. Príprava spermií a testikulárneho tkaniva pre imunohistochémiu a Western blot

Testikulárne tkanivo bolo odobraté z kancov po prepláchnutí zvieraťa studeným PBS. Zviera sa najprv uspalo pomocou zmesi TKX (Tiletamin 4 mg/kg, Zolazepam 4 mg/kg (Zoletil 100, Virbac), Ketamin 5 mg/kg (Narketan 10, Chassot) a Xylazin 1 mg/kg (Rometar 2%, Spofa)). Dávkovanie zmesi je 1 ml na 15 kg. Po uspaní bol následne do žily podaný heparín (Heparin inj. 1x10ml/50KU). Nasledovalo prepláchnutie zvieraťa studeným PBS vychladeným na 4 °C. Po vybratí semenníka, bol pravý semenník fixovaný v 4% paraformaldehyde s následnou kryoprotekciou v 30% sacharóze a v 0,1 M PBS. Takto pripravené tkanivo sa následne využívalo na imunohistochémiu a elektrónovú mikroskopiu. Ľavý semenník sa použil na SDS-PAGE a Western blot.

Zmrazené testikulárne tkanivo bolo krájané pomocou kryostatu Leica CM1950. Rezy semennikov s hrúbkou 5 μm boli namontované na podložné sklíčka potiahnuté 2% siláne (Sigma-Aldrich). Sklíčka sa zahrievali 10 minút pri pretlaku 0,7 bar v 0,01 M pufru citrátu sodného (pH 6,0) s použitím tlakového hrnca (Steba, Nemecko) na získanie antigénu. Rezy boli blokované 10% kozím sérom. Následne bola použitá monoklonálna myšacia protilátka anti-PCNA (ab29, Abcam 1:2000) a monoklonálna králičia protilátka anti – Ki67 (ab 16667, Abcam 1:1000) alebo králičia anti-Htt monoklonálny protilátka (EPR5526, Abcam 1:250) cez noc pri teplote 4°C. Rezy farbené EPR5526 sa ďalej spracovali s Alexa konjugovanou koziou protilátkou (Amersham 1: 500) počas 1 hodiny pri laboratórnej teplote. Následne došlo k montovaniu rezov s montážnym médiom obsahujúcim DAPI. Rezy boli digitalizované pomocou skenovacieho mikroskopu Olympus BX a obrázky boli upravené pomocou softvéru VS-120.

SDS-PAGE a Western Blot

Semenníky sa homogenizovali v kvapalnom dusíku za pomoci mažiaru. Homogenizované semenníky boli lyzované v RIPA pufri (150nM NaCl,1 % NP – 40, 0,5 % deoxycholát, 0,1 % SDS, 50nM Tris-HCl pH8, inhibítory fosfatáz a proteáz). Následne sa homogenát sonifikoval a centrifugoval pri 4 °C, 10 000G počas 10 minút. Vzorky (20 μg celkového proteínu) boli nanesené na 3-8% Tris-acetátový gél (EA03755, LifeTech). Rozdelenie proteínov prebiehalo

pri 150 V. Gél sa preniesol na nitrocelulózovu membránu (IB301001, LifeTech) pri 250 mA. Membrána bola blokovaná v 5 % odstredenom mlieku. Následne bola pridaná anti Htt protilátka (EPR5526, Abcam, 1: 30 000 alebo AB1, Sigma Aldrich; 1 000) alebo anti-polyQ protilátka (3B5H10, Sigma Aldrich; 1: 3 000). Ako kontrola sa použilo farbenie tubulínom (anti-tubulin; Sigma Aldrich; 1: 10 000). Následne sa použila konjugovaná sekundárna protilátka s HRP (anti-myšacia, 711-035-152, Jackson ImmunoResearch; 1: 10 000 alebo anti-králičia, 711-035-152; Jackson ImmunoResearch; 1: 10 000). Signál bol zachytený na filmoch CL-Xposure (34091, Thermo Scientific) a vyvolaný pomocou ECL (RPN2232; GE Healthcare).

Na štatistickú analýzu sa používal program GraphPad Prism 5.0. Vzorky sa štatisticky vyhodnocovali pomocou ANOVA testu a Ducanovho testu.

3.4. Príprava vzoriek pre hormonálnu analýzu

Hormonálny test

Vzorky krvi sa v priebehu štúdie odobrali 5 krát. Na pokus sa použilo 15 TG a 8 WT kancov zodpovedajúceho veku (7 – 30 mesiacov). Vzorky krvi sa nechali zrážať 60 minút pri laboratórnej teplote. Nasledovala centrifúgacia pri 1500 G, 10minút pri 4 °C. Odsali sme supernatan a ten sme centrifugovali 1500 G, 10minút pri 4 °C. Sérovéhladiny testosterónu a luteinizačného hormónu (LH) boli stanovené pomocou komerčných súprav. Súpravy ELISA (CSB-E06796p, CSB-E06791p, CSB-E12870p, CSB-EL-011718PI; CUSABIO, Wuhan, Čína).

Na štatistickú analýzu sa používal program GraphPad Prism 5.0. Vzorky sa štatisticky vyhodnocovali pomocou Kolmogorov-Smirnov test normality a nepárový T testom.

3.5. Príprava spermií pre transmisnú elektronovú mikroskopiu, vyhodnotenie vzoriek

Malé bloky vzoriek testikulárneho tkaniva a ejakulátu boli fixované v 300 mM glutaraldehydu (Sigma-Aldrich) v 100 mM kakodyláte pufru počas 2 hodín pri izbovej teplote (RT), premytý rovnakým pufrom a postfixované v 40 mM oxidu osmičelého (Polysciences) pri 100 ° C mM kakodylátového tlmivého roztoku počas 1 hodiny pri izbovej teplote. Vzorky testikulárneho tkaniva boli vložené do aralditovej živice (Durcupan ACM, Sigma-Aldrich) opláchnuté v kakodylátovom tlmivom roztoku a dehydratované v etanole. Vzorky ejakulátu boli vložené do

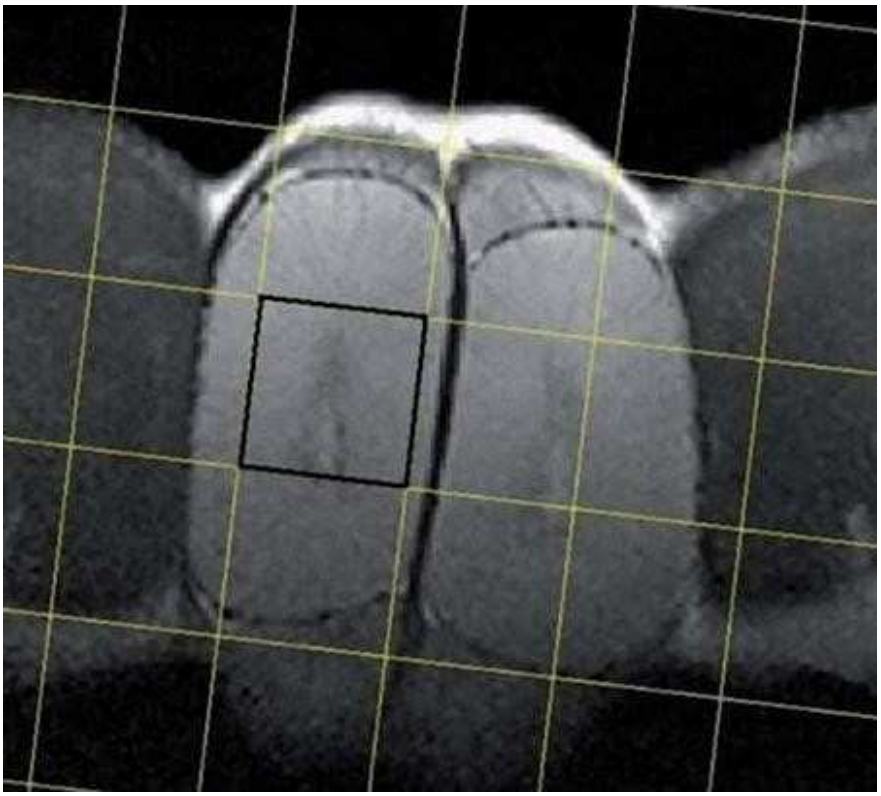
agarových blokov, dehydratované v etanole a vložené do aralditovej živice (Durcupan ACM, Sigma-Aldrich).

Pre imunohistochemickú analýzu boli vzorky ejakulátu premyté sa v PBS a fixované v 4% paraformaldehyde s 0,1% glutaraldehydu v PBS. Vzorky boli vložené do agarových blokov, dehydratované v etanole a zapustené do LR bielej živice (Sigma-Aldrich). Vzorky sa inkubovali s myšou anti-polyglutamínovou monoklonálnou primárnou protilátkou (MAB1574, Millipore, 1: 50) cez noc pri 4 ° C. Potom boli rezy prpláchnuté v PBS a inkubované s protilátkou proti myšaciemu IgG-Gold (10 nm zlaté častice; G7652; Sigma-Aldrich; 1:40) počas 2 hodín pri izbovej teplote. Vo všetkých pre elektrónovú mikroskopiu sa použili rezy s hrúbkou 60 nm narezané pomocou ultramikrotomu Leica EM UC6 a zafarbené uránom acetátom s citrátom. Sekcie boli preskúmané pod FEI Morgagni 268D elektrónovým mikroskopom (spoločnosť FEI, Holandsko) pri 70 ° C kV.

3.6. Príprava kancov pre ^{31}P MR spektroskopiu

Pre pokus bolo použitých 5 TgHD kancov a 5 WT kancov z F2 generácie. Kance boli pod anestéziou. Ako anestetikum bol použitý propofol (Propofol 1% Fresenius) v kombinácii s itramuskulárnou premedikáciou aplikáciou zmesi TKX (Tiletamin 4 mg/kg, Zolazepam 4 mg/kg (Zoletil 100, Virbac), Ketamin 5 mg/kg (Narketan 10, Chassot) and Xylazin 1 mg/kg (Rometar 2%, Spofa)) v kombinácii s diazepanom (0.25 mg/kg, Apaurin). Na kontrolu životných funkcií bol na chvost pripnutý pulzný oximeter.

^{31}P MR spektoroskopia bola vykonávaná za použitia prístroja 3T MR skaneru (Siemens Magnetom Trio). Vonkajšia dvojitá povrchová cievka 1H / 31P (Rapid Biomedical, Rimpar, Nemecko) bola umiestnená pod zviera v brušnej polohe. Pre lokalizáciu spektra boli tri kolmé obrazy získané štandardnou sekvenciou T2W echoturbospin (TR = 4 400 ms, TE = 99 ms, hrúbka rezu = 4 mm), spektrá boli získané sekvenciou 2D - CSI (Two - Dimensional ChemicalShift Imaging) (TR = 4 000 ms, TE = 2ms, VOI 240x240x25 mm)



Obr.č. 11 **Pozície spektroskopickej mriežky v programe jSIPRO**

(obrázok z publikácie (Jozefovicova et al., 2016))

Na štatistickú analýzu sa používal program GraphPad Prism 5.0. Vzorky sa štatisticky vyhodnocovali pomocou Shapiro-Wilk test na normálnu distribúciu. Následne bol použitý Studentov –T test.

3.7. Príprava spermií pre štúdium metabolizmu mitochondrií

Vzorky spermií sa získali od TgHD kancov a ich kontrolných WT súrodencov (n = 12) z dvoch generácií (Fl a F2) vo veku medzi 12 a 65 mesiacmi. Vzorky boli získané opakovane počas trojročného priebehu štúdie až osemkrát od jedného zvieraťa. V týchto vzorkách bola stanovená MM (vrátane cyklu trikarboxylovej kyseliny (TCA), OXPHOS a komplexu pyruvát dekarboxylázy (PDHc) detekciou oxidácie rádioaktívne značených substrátov (MEGS) a obsah podjednotiek OXPHOS bol detegovaný Western blotom. U ôsmich kancov bolo meraná respirácia spermií v časovom priebehu troch rokov. Vždy boli porovnávené dvojice súrodencov TgHD a WT.

Pair No.	HD status	generation	age at the time of measurement (months)
1	TgHD	F1	12, 14, 19, 23, 30, 36, 42, 47
1	WT	F1	22, 24, 29, 33, 41, 46, 53, 58
2	TgHD	F1	12, 14, 19, 23, 30, 36, 42, 47
2	WT	F1	40, 48, 53, 60, 65
3	TgHD	F2	14, 18, 22, 23, 30, 34
3	WT	F2	14, 18, 22, 23, 30, 34
4	TgHD	F2	22, 25, 32, 36
4	WT	F2	20, 22, 25, 32, 36
5	TgHD	F2	16
5	WT	F2	16
6	TgHD	F2	16
7	WT	F1	10, 12

Obr.č. 12 ***Plán odberu vzoriek pre meranie MEGS***

(obrázok z publikácie (Krizova et al., 2017))

Pair No.	HD status	generation	age at the time of measurement (months)
1	TgHD	F1	30, 36, 42, 47
1	WT	F1	41, 46, 53, 58
2	TgHD	F1	30, 36, 42, 47
2	WT	F1	48, 53, 60, 65
3	TgHD	F2	20, 23, 30, 34
3	WT	F2	20, 23, 30, 34
4	TgHD	F2	22, 25, 32, 36
4	WT	F2	22, 25, 32, 36

Obr.č. 13 ***Plánodberu vzoriek pre respiračné analýzy***

(obrázok z publikácie (Krizova et al., 2017))

Odber semena a izolácia spermií

Všetky ejakuláty sa vyhodnotili pomocou SCA analyzátora (Microptic, Spain) ihneď po odbere. Časť čerstvého ejakulátu bohatého na spermie sa zriedil 2:1 s BTS riedidlom. Následne boli ponechané v kľude pri izbovej teplote počas 30 minút. Potom vzorka bola centrifugovaná počas 20 minút pri 25 ° C a 250 G.. Bunky boli opäť premyté v pomere 1: 2 a 1: 1 s BST a táto suspenzia bola použitá na analýzy. Funkčné merania (respiračné a MEGS analýzy) boli vykonané počas jedného dňa. Počas prepravy boli premyté bunky inkubované pri izbovej teplote pre meranie dýchania. Pri analýze kapacity mitochondriálneho systému generovania energie (MEGS) sa spermie prepravovali a uchovávali na mokrom ľadu a pre

analýzy proteínov / WB sa bunky ihneď zmrazili v kvapalnom dusíku a uskladnili sa pri teplote -80 ° C až do použitia.

Meranie kapacity mitochondriálneho systému generujúceho energiu (MEGS)

Z homogenátu čerstvých spermií (celkový počet 6 až 32 x 10^9 spermií) sa pripravil pri 4 °C v sklenenom homogenizátore Potter-Elvehjem v 150 mM KCI, 50 mM Tris-HCl, 2 mM EDTA, pH 7,4 s prídavkom proteázového inhibičného koktailu (PIC, Sigma-Aldrich, Saint Louis, MO, USA). Homogenát sa sonifikoval trikrát počas 5 s a použil sa na analýzu MEGS.

Kapacita MEGS sa analyzovala v čerstvom homogenáte meraním oxidačných pomerov [^{14}C] pyruvátu, [U-^{14}C] malátu a [1,4-^{14}C] sukcinátu podľa Janssena (Janssen, Trijbels a kol., 2006) v desiatich rôznych inkubáciách. Pre každú reakciu sa použilo 5 ul homogenátu s koncentráciou proteínu 4 až 8 mg / ml. Produkcia ^{14}CO2 sa spočítala v Beckman Coulter LS (Brea, CA, USA).

Respirometria

Spotreba kyslíka bola meraná v permeabilizovaných spermiách s digitonínom pri 37 ° C pomocou OROBOROS Oxygraph-2k (OROBOROS INSTRUMENTS Corp, Innsbruck, Rakúsko) v 2ml komore s médiom na báze KCl (80mM KCI, 10mM Tris, 3mM MgCl2, 1mM EDTA, 5 mM fosforečnan draselný, pH 7,4). Permeabilizácia plazmatickej membrány bola uskutočnená ručnou titráciou približne 4,1 µM digitonínu. Nasledujúci protokol zahŕňal viaceré substráty a inhibítory 10 mM glutamát, 2,5 mM malát, 1 mM ADP + Mg2 +, 0.5 pM rotenón, 10 mM sukcinát, antimycín A, 4mM askorbát a 0,4mM TMPD (N, N, N ', N'-tetrametyl-1,4-fenyléndiamín) Dýchanie bolo uvoľnené titráciou 1,5 uM FCCP (karbonylkyanid p (trifluórmetoxy) fenylhydrazón) a nakoniec sa inhibovalo pridaním 10 mM azidu sodného.

Rozdiely medzi pozorovanými aktivitami / pomermi enzýmov v kontrolách TgHD a WT s ohľadom na vek a generáciu sa skúmali pomocou modelov lineárnej regresie so zmiešanými účinkami. Hodnoty P (upravené pre viacnásobné porovnanie pomocou metódy Benjamini & Yekutieli) menej ako 0,05 sa považovali za štatisticky významné. Analýzy boli vykonané s použitím štatistického balíka R, verzia 3.2.2.

SDS-PAGE a imunodetekcia mitochondriálnych proteínov

Spermie boli lyzované v pufri RIPA (50 mM Tris-HCl (pH 7,4), 150 mM chloridu sodného, 1 mM PMSF, 1 mM EDTA, 1% Triton X-100 a 0,1% SDS, PIC (Sigma-Aldrich, Saint Louis, MO, USA)) počas 20 minút na ľade a centrifugované pri 51 000 G počas 25 minút pri teplote 4 ° C. Vzorky sa disociovali vo vzorkovacom pufri obsahujúcom 50 mM Tris-HCl (pH 6,8), 12% (V / V) glycerolu, 4% SDS, 2% (V / V) 2-merkaptoetanol a Bromphenol Blue 0,01% počas 30 minút, 37 ° C. Proteíny boli separované na 12% polyakrylamide. Do každej dráhy bolo nanesených 10 ug proteínu.

Proteíny boli elektroblotované na membránach Immobilon®-P PVDF (Millipore, Billerica, MA, USA) s polosuchým prenosom počas 1 hodiny pri konštantnom prúde (0,7 mA / cm2). Membrány vysušené vzduchom (cez noc) boli opláchnuté v 100% metanole a blokované 5% roztokom sušeného mlieka bez tuku v TBS-pufrovanom fyziologickom roztoku (TBS) počas 2 hodín. Bloty boli inkubované s primárnymi protilátkami v 0,1%) Tween 20 a 1% roztoku sušeného mlieka 2 hodín. Pre homeostázu mitochondriálneho metabolizmu boli použité nasledovné monitorovacie protilátky: anti-SDHA (ab14715), anti-SDHB (ab14714), anti-UQCR1 (ab110252), anti-NDUFA9 (ab14713), anti-NDUFB6 (ab110244)) Abbot, protilátka anti-FO-OSCP (ab110276), Anti-Aconitáza 2 (ab110321) (všetky uvedené vyššie od Abcam, Cambridge, UK), anti-OPA1 (612606, BD biosciences, San Jose, CA, USA) a anti-β-tubulín (T4026, Sigma-Aldrich, Saint Louis, MO, USA). Ako sekundárna protilátka sa použila IgG peroxidázovo konjugovaná anti myšacia alebo anti králičia protilátka (A8924, A0454, ako Sigma-Aldrich, Saint Louis, MO, USA) v 0,1% Tween 20 a 1% roztoku mlieka počas 1 hodiny. Vizualizácia bola uskutočnená pomocou SuperSignal West Femto maximálneho citlivého substrátu (ThermoFisher Scientific, Waltham, MA, USA) na systéme zobrazovania SynGene (Synoptics Group, Cambridge, UK).

SDS-PAGE a imunodetekcia huntingtinu

Vzorky zmrazených tkanív boli lyzované v RIPA pufri pre spermie (150 mM NaCl, 0,05% NP-40, 1% deoxycholát sodný, 0,1% SDS, 1% Triton X-100, 5mM EDTA pH 8.0, 50mM Tris- HCl pH 8,0, močovina, DTT, inhibítory fosfatáz a proteáz). Potom sme ich vortexovali najmenej 60 minút pri 4 ° C, následne sa sonifikovali počas 15 minút a centrifugovali sa pri 15 000 G počas 10 minút pri 4 ° C. Vzorky boli nanesené na 3- 8% Tris-acetátový gél (ThermoFisher Scientific, Waltham, MA, USA) a gél bol napojený pri 150 V. Gél bol prenesený na nitrocelulózovú membránu (ThermoFisher Scientific, Waltham, MA, USA) ,

membrány boli zablokované v 5% sušenom mlieku počas 1 hodiny a sondy boli cez noc testované vhodnou protilátkou. Pre naše experimenty sme použili anti-Htt protilátku (EPR5526, Abcam, Cambridge, UK, 1: 3000). Bola použitá sekundárna peroxidázou konjugovaná protilátka (anti-králičie IgG, Jackson ImmunoResearch, West Grove, PA, USA, 1: 10000, # 711-035-152).

4. VÝSLEDKY

Monika Macakova, **Bozena Bohuslavova**, Petra Vochozkova, Antonin Pavlok, Miroslava Sedlackova, Daniela Vidinska, Klara Vochyanova, Irena Liskova, Ivona Valekova, Monika Baxa, Zdenka Ellederova, Jiri Klima, Stefan Juhas, Jana Juhasova, Jana Klouckova, Martin Haluzik, Jiri Klempir, Hana Hansikova, Jana Spacilova, Ryan Collins, Ian Blumenthal, Michael Talkowski, James F. Gusella, David S. Howland, Marian DiFiglia, Jan Motlik (2016)**Mutated Huntingtin Causes Testicular Pathology in Transgenic Minipig Boars**. *Neurodegenerative Diseases*, *16*(3–4), 245–259. https://doi.org/10.1159/000443665

IF (2016) 2,842

M. Jozefovicova, V. Herynek, F. Jiru, M. Dezortova, J. Juhásová, S. Juhas, J. Klíma, **B. Bohuslavova,** J. Motlik, M. Hájek (2015) **^{31}P MR Spectroscopy of the Testes and Immunohistochemical Analysis of Sperm of Transgenic Boars Carried N-terminal Part of Human Mutated Huntingtin,** *Česká a slovenská neurologie a neurochirurgie*, Cesk Slov Neurol N 2015; 78/111(Supplementum 2): 28-33,*DOI:* 10.14735/amcsnn20152S28

IF (2015) 0,209

Jana Krizova, Hana Stufkova, Marie Rodinova, Monika Macakova, **Bozena Bohuslavova**, Daniela Vidinska, Jiri Klima, Zdenka Ellederova, Antonin Pavlok, David S. Howland, Jiri Zeman, Jan Motlik and Hana Hansikova *(2017),* **Mitochondrial metabolism in large-animal model of huntington's disease: the hunt for biomarkers in the spermatozoa of presymptomatic minipigs** *Neuro-Degenerative Diseases*, *17*(4–5), 213–226. https://doi.org/10.1159/000475467

IF (2017) 2,842

4.1. Mutovaný huntingtín spôsobuje testikulárnu patológiu u kancov transgeného miniprasaťa

Mutated huntingtin causes testicular pathology in transgenic minipig boars

Monika Macakova, **Bozena Bohuslavova**, Petra Vochozkova, Antonin Pavlok, Miroslava Sedlackova, Daniela Vidinska, Klara Vochyanova, Irena Liskova, Ivona Valekova, Monika Baxa, Zdenka Ellederova, Jiri Klima, Stefan Juhas, Jana Juhasova, Jana Klouckova, Martin Haluzik, Jiri Klempir, Hana Hansikova, Jana Spacilova, Ryan Collins, Ian Blumenthal, Michael Talkowski, James F. Gusella, David S. Howland, Marian DiFiglia, Jan Motlik (2016)

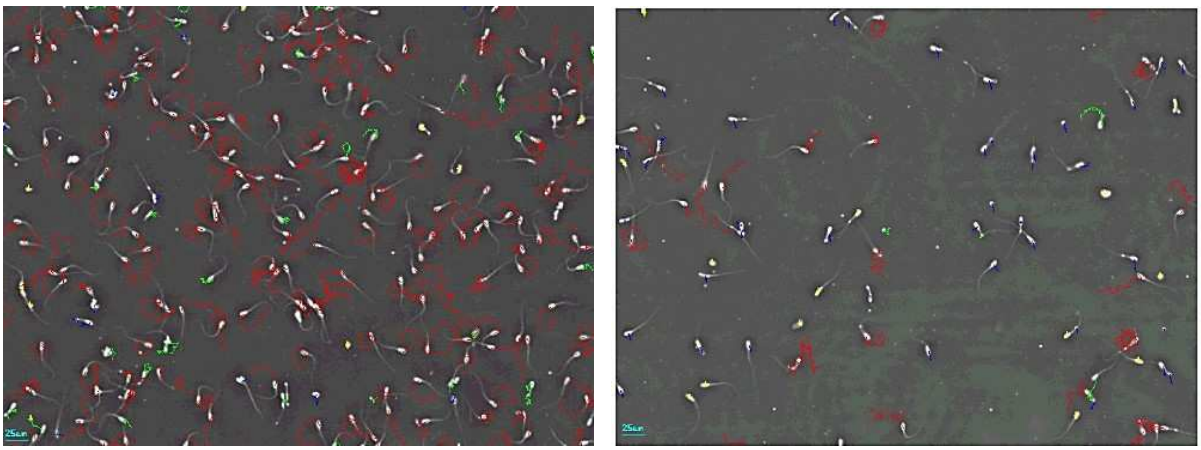
Neurodegenerative Diseases, *16*(3–4), 245–259. https://doi.org/10.1159/000443665

IF (2016) 2,842

V tejto publikácii poukazujeme na patológiu TgHD semenníkov kancov v dôsledku prítomnosti mutovaného proteínu huntingtínu. Aj keď mtHtt spôsobuje predovšetkým degeneráciu neurónov, môže ovplyvňovať aj periférne tkanivá. U F1 generácie našich TgHD kancov sa ako prvý fenotyp prejavil práve rozvoj testikulárnej degenerácie – a to hlavne ako poruchy v reprodukcii. Prvýkrát bol pozorovaný vo veku 13 mesiacov kancov (Baxa et al.,2013). Môže to byť spôsobené práve tým, že semenníky majú najviac podobnú génovú expresiu ako mozog a táto podobná expresia môže spôsobiť atrofiu semenníkov, ktorá bola taktiež pozorovaná u trangénnych modeloch HCH myší.

Cieľom publikácie bolo sledovanie reprodukčných parametrov TgHD kancov v porovnaní s ich WT súrodencami. Na testovanie sme použili najstarších kancov z F1 a F2 generácie (rok narodenia 2009-2011). Zamerali sme sa na patológiu spermií a semenníkov. Prvotnou úlohou bolo zistiť, či inzert lentivírusového konštruktu neporušil žiadne kódujúce sekvencie v genóme prasaťa. Potom sme sa zamerali na poruchu reprodukcie. Počet spermií v ejakuláte, progresivitu a motilitu spermií sme detegovali pomocou SCA analyzátora (Sperm Cell Analyzer). Tam sme dokázali, že TgHD kance majú znížený počet spermií (obr.č. 14, 15), zníženú progresivitu (obr.č. 17) a motilitu (obr.č. 14, 16). Pri penetračných testoch sme zistili zníženú schopnosť penetrovať do oocytu *in vitro* u spermií TgHD kancov (obr.č. 18). Všetky parametre sa s pribúdajúcim vekom zhoršovali. Pomocou elektrónovej mikroskopie boli

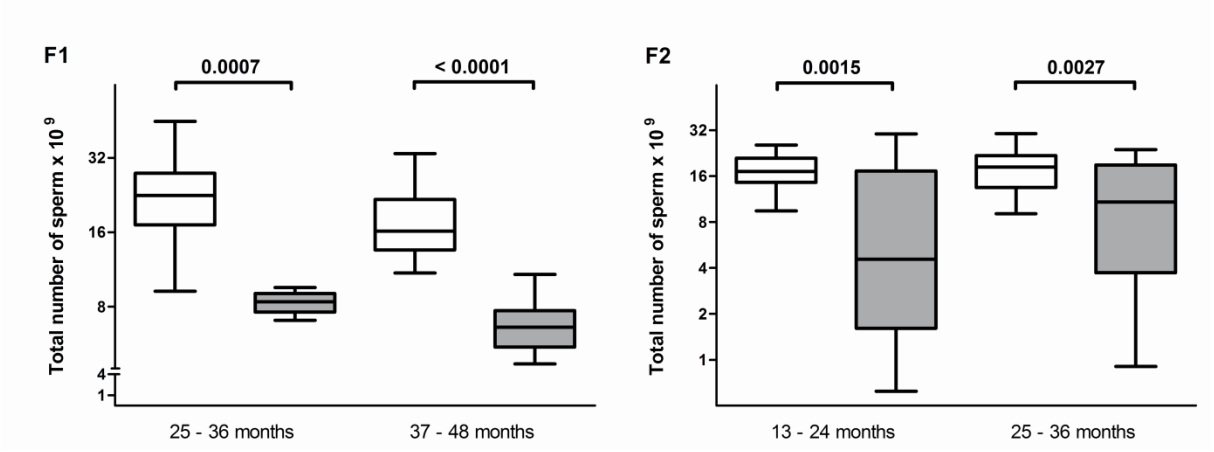
detegované mnohé patologické zmeny bičíkov (deformácia mitochondriálneho plášťa bičíka a krčka spermie, zvinuté bičíky, dvojitá axonéma) (obr.č. 19) a tiež epitelu semenníkov (Sertoliho bunky boli apoptotické, čo sa prejavilo zvýšenou denzitou cytoplazmy, jej vakualizáciou) (obr.č. 20)



A B

Obr.č. 14 ***Počet spermií a motilita*** *A) V ľavo vidíme vzorku ejakulátu WT kanca, kde je výrazne vyšší počet spermií a motilita oproti vzorke TgHD kanca napravo, B) Vzorka ejakulátu TgHD kanca, červená farba označuje veľmi rýchle spermie, zelená farba stredne rýchle spermie, modrá farba pomalé spermie a žltá nepohyblivé spermie)(zdroj Centrum PIGMOD – nepublikovaný obrázok)*

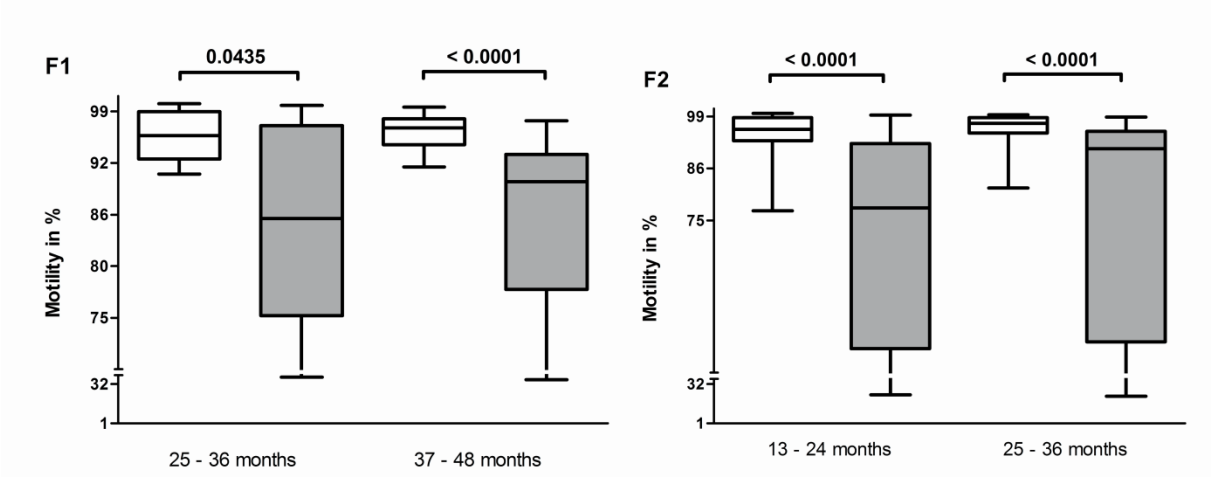
Okrem toho imunohistochemické vyšetrenie potvrdilo signifikantne nižšiu proliferačnú aktivitu spermatogónií v semenníkoch TgHD kancov. V štúdii sme poskytli dôkaz degenerácie spermií a testikulárnej dysfunkcie na modely transgenného miniprasaťa pre HCH. Boli detegované fragmenty mtHtt, ktoré indukujú cytotoxicitu (obr.č. 21). V spermiách bol exprimovaný endogénny aj mutovaný huntingtín (obr.č. 21).



A B

Obr.č. 15 ***Počet spermií*** *A) Počet spermií TgHD kancov z F1 generácie vo veku 25 -36 mesiacov a vo veku 37-48 mesiacov bol signifikatne znížený v porovnaní z kontrolnými zvieratami, B) počet spermií v ejakuláte TgHD kancov z F2 generácie je tiež nižšia oproti WT súrodencom. Na analýzu bol použitý Kolmogorov-Smirnov test normality a štatistické vyhodnotenie Mann-WhitneyU test.*

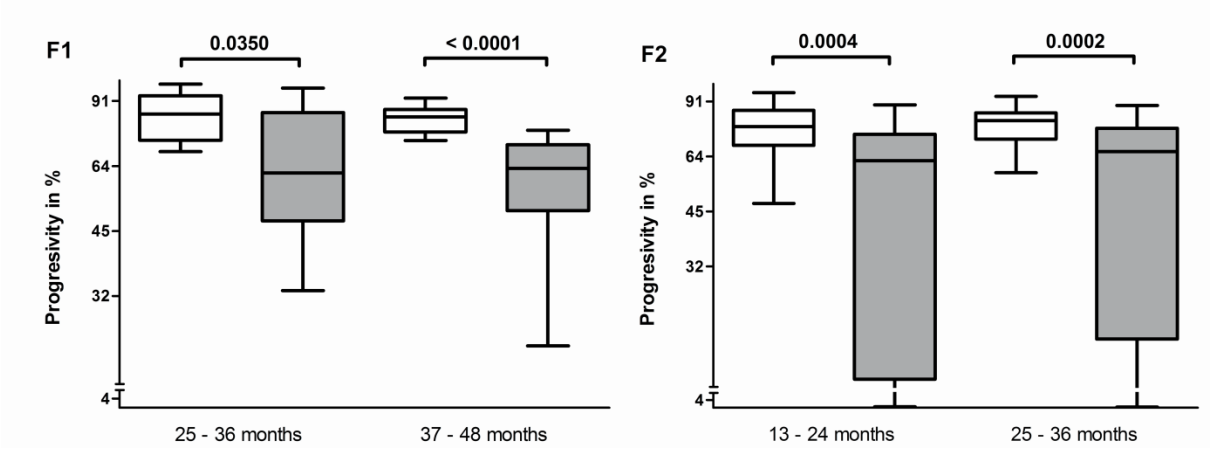
(z Macakova, Bohuslavova et al., 2016)



A B

Obr.č. 16 ***Motilita spermií*** *A) Signifikatne znížená motilita spermií TgHD kancov z F1 generácie vo veku 37-48 mesiacov B) Signifikantne znížená motilita spermií TgHD kancov z F2 generácie vo veku 13-24 mesiacov a vo veku 25 -36 mesiacov. Na analýzu bol použitý Kolmogorov-Smirnov test normality a štatistické vyhodnotenie Mann-WhitneyU test.*

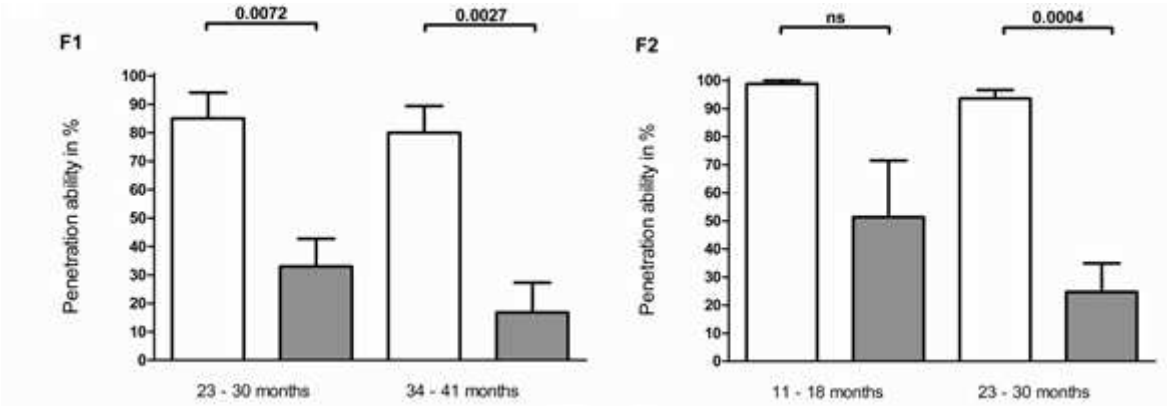
(z Macakova, Bohuslavova et al., 2016)



A B

Obr.č. 17 ***Progresivita spermií*** *A) Signifikantne znížená progresivita spermií TgHD kancov u F1 generácie vo veku 37 -48 mesiacov. B) Znížená progresivita spermií TgHD kancov u F2 generácie. Na analýzu bol použitý Kolmogorov-Smirnov test normality a štatistické vyhodnotenie Mann-WhitneyU test.*

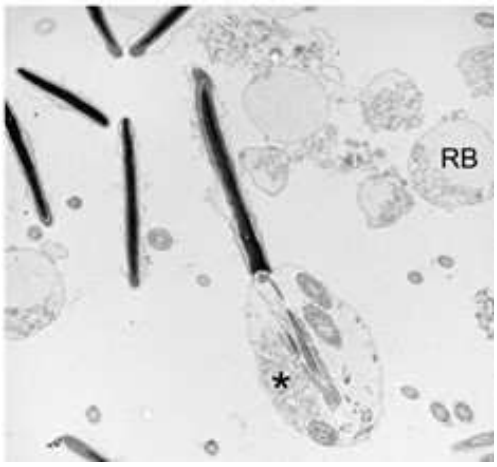
(z Macakova, Bohuslavova et al., 2016)

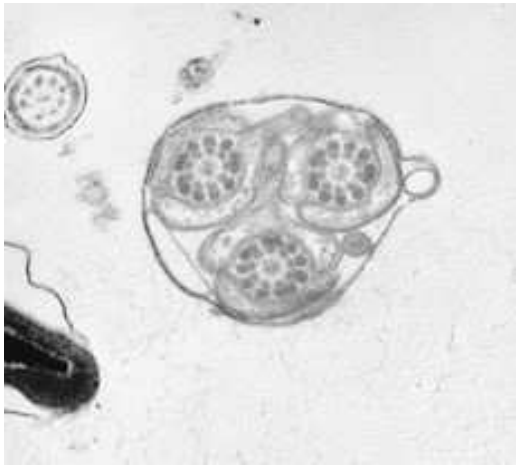


A B

Obr.č. 18 ***Znížená penetračná schopnosť spermií*** *A) Signifikante znížená penetračná schopnosť spermií TgHD F1 kancov vo veku 23-30 mesiacov a vo veku 34-41 mesiacov. B) Znížená penetračná schopnosť spermií TgHD F2 kancov vo veku 11-18 meseiacov a vo veku 23-30 mesiacov. Na analýzu bol použitý Kolmogorov-Smirnov test normality a štatistické vyhodnotenie Mann-Whitney U test.*

(zdroj Centrum PIGMOD – nepublikovaný obrázok)

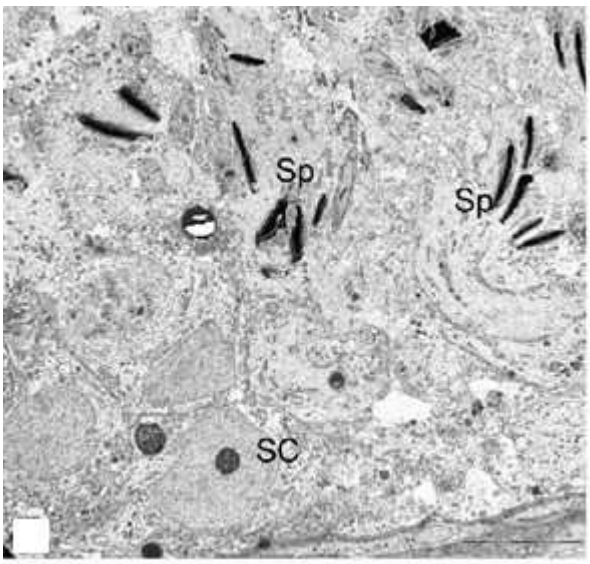




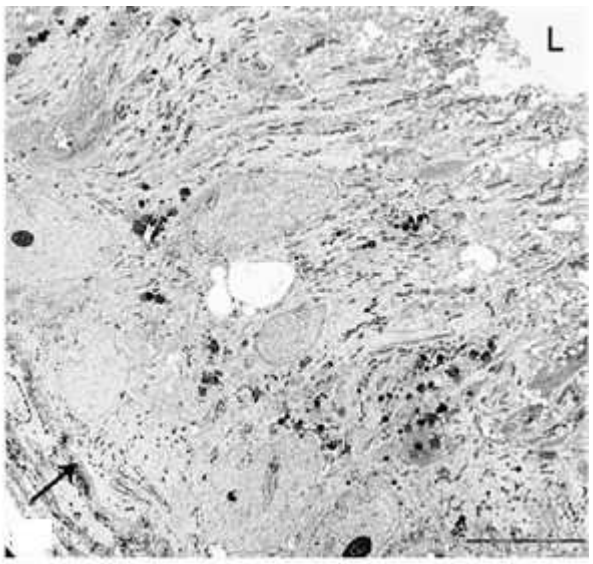
A B

Obr.č. 19 ***Morfologické zmeny spermií*** *A) Spermia s cytoplazmatickou kvapkou B) Bičík s trojitou axonémou*

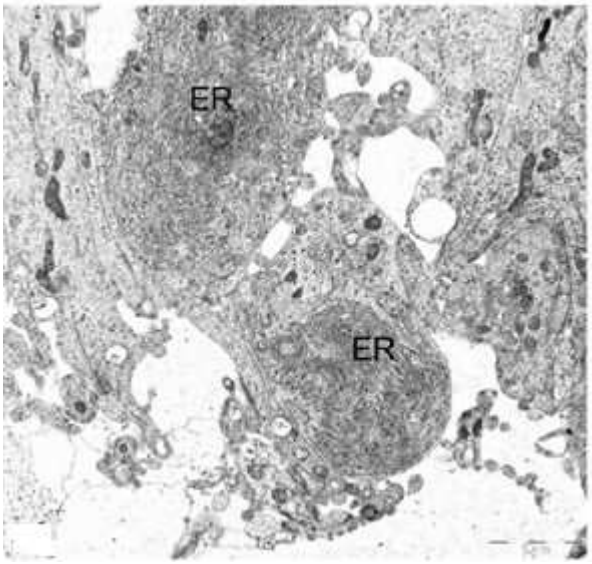
(z Macakova, Bohuslavova et al., 2016)



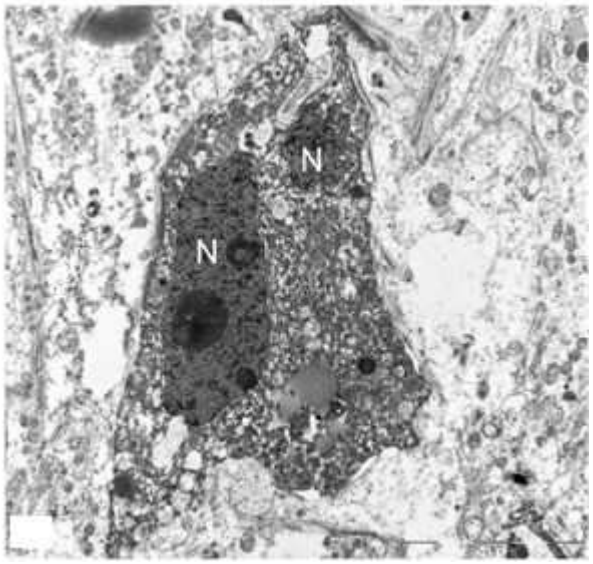
A



B



C

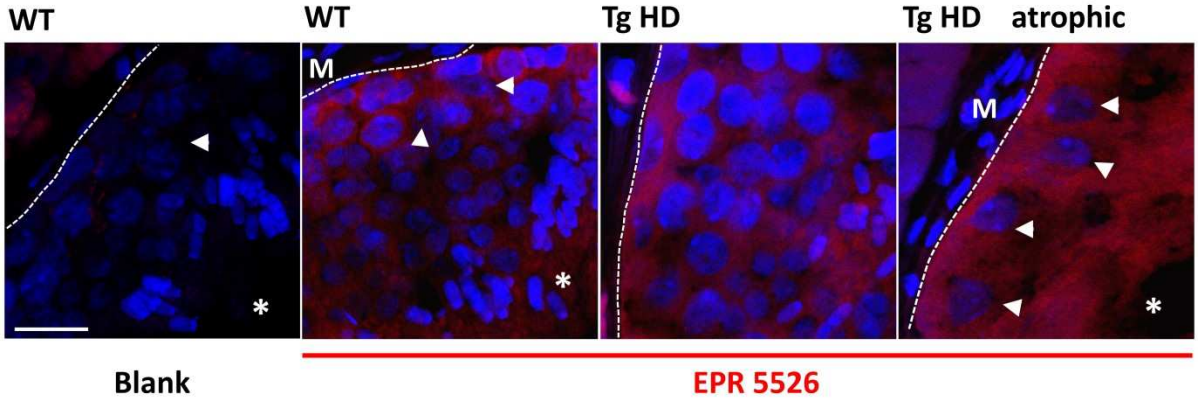


D

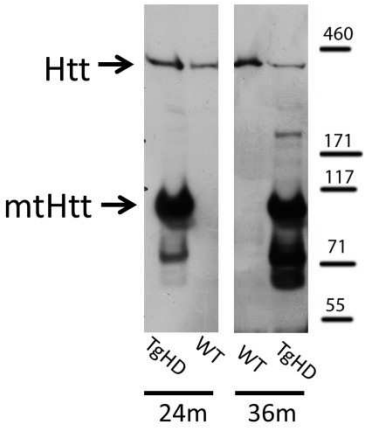
Obr.č. 20 ***Semenotvorný epitel TgHD kancov vo veku 36 mesiacov****. A- Znížená spermatogenézy. Sertoliho buniek (SC), spermií (Sp). (B) semenotvorný epitel obsahujúci iba intaktné Sertoliho bunky, žiadne prvky spermatogenézy*

(C) Sertoliho bunky toho istého tubulu - sústredné cisterny endoplazmatického retikulum (ER) v apikálnej cytoplazme. (D) Sertoliho buniky podstupujúcich apoptózu v kanáliku. Jadro Sertoliho bunky (N).

(z Macakova, Bohuslavova et al., 2016)



A



B

Obr.č. 21 ***Expresia Htt v semenníkoch*** *A) M = myoidné bunky. Celkový proteín huntingtínbol imunohistochemicky vizualizovaný pomocou protilátky EPR5526 v sekciách WT a transgénnych (TgHD) prasacích semenníkov. EPR5526 (červená, farba) bola detegovaná v semenotvornom epiteli ohraničenom membránou zo základnej membrány (čiarka) a lumenom (hviezdičkou). Myoidné bunky boli negatívne zafarbené. Bunkové jadrá boli kontrastné s DAPI (modrá). Významné jadrá Sertoliho buniek (šípky) prítomné v atrofických semenných tubulách (atrofické TgHD) dokumentovali stratu zárodočných buniek. Prázdna sekcia WT testis zafarbená iba s sekundárnou protilátkou konjugovanou s A647. Stupnica s mierkou = 20 µm. B) Western analýza semenníkov; Reprezentatívna vzorka. Protilátka detegovala endogénnu Htt, transgénnu mtHtt a fragmenty.*

(z Macakova, Bohuslavova et al., 2016)

Touto štúdiou sme preukázali poškodenie spermií TgHD kancov a rozsiahlu testikulárnu degeneráciu na modely TgHD miniprasaťa. Dokázali sme, že inzercia lentivírusového konštruktu neporušila žiadnu kódujúcu sekvenciu v genóme prasaťa. Štúdia naznačila, že testikulárny defekt bol spôsobený prítomnosťou mtHtt a jeho fragmentovanej cytotoxickej formy v testikulárnom tkanive, pretože sme nezaznamenali hormonálne zmeny, ktoré sme merali u TgHD zvierat aj u ich zdravých súrodencov.

4.2. ^{31}P MR spektroskopia semenníkov a imunohistochemická analýza spermií transgénnych kancov realizovaná pre N koncovú časť ľudského mutovaného huntingtinu

^{31}P MR Spectroscopy of the Testes and Immunohistochemical Analysis of Sperm of Transgenic Boars Carried N terminal Part of Human Mutated Huntingtin

M. Jozefovicova, V. Herynek, F. Jiru, M. Dezortova, J. Juhásová, S. Juhas, J. Klíma, **B. Bohuslavova,** J. Motlik, M. Hájek

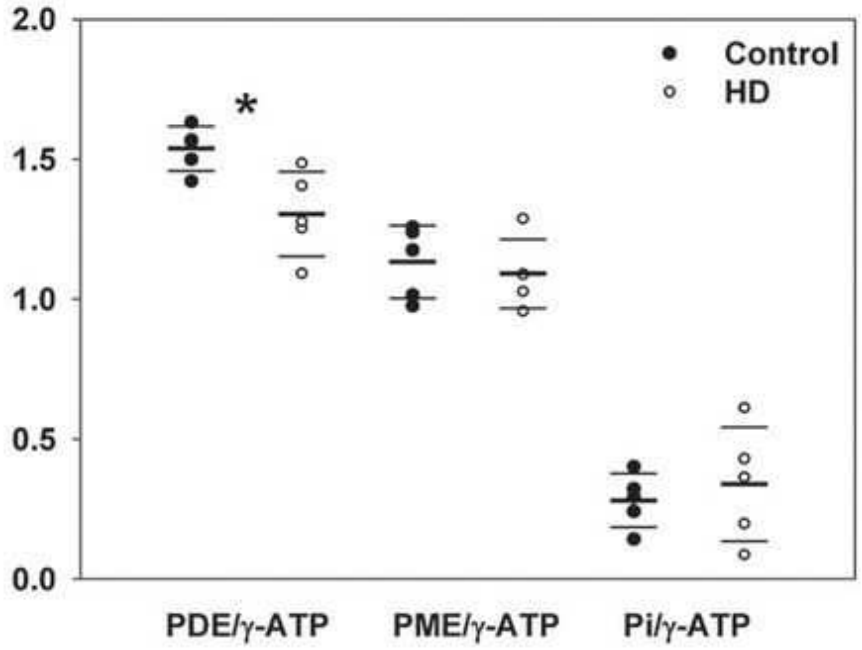
Článek: Cesk Slov Neurol N 2015; 78/111(Supplementum 2): 28-33
DOI: 10.14735/amcsnn20152S28
IF 0,209

Cieľom tejto štúdie bolo preukázať zmeny v semenníkoch 24 mesačných TgHD kancov F2 generácie pomocou ^{31}P magnetickej rezonančnej spektroskopie a pomocou imunohistochemickej analýzy TgHD spermií odobraných z F1 a F3 generácie pred prejavením sa klinických príznakov HCH.

^{31}P MR spektroskopia je neinvazívna metóda, ktorú možno použiť na monitorovanie metabolických zmien v rôznych typoch tkanív (mozog, pečeň, sval, semenníky). *In vivo* ^{31}P MR spektrum zo semenníkov obsahuje sedem charakteristických píkov pochádzajúcich z fosfomonoesterov (PME), fosfodiesterov (PDE), adenosíntriphosfátov (tri píky predstavujú tri fosfáty – ATP - α, -β, -γ), anorganického fosfátu (Pi) a fosfokreatínu (PCr). Fosfokreatín nie je prítomný v semenníku, ale je ako zvyšok po kontaminácii zo svalu.

Na ^{31}P MR spektroskopiu bolo použitých 5 TgHD kancov a 5 WT súrodencov z F2 generácie vo veku 2 rokov. Na študovanie spermií pomocou imunohistochemickej analýzy boli použité spermie z 1TgHD a 1WT zvieraťa z F1 generácie vo veku 4 -5 rokov, a z 1 TgHD a 1 WT kanca z F3 generácie vo veku 2 rokov.

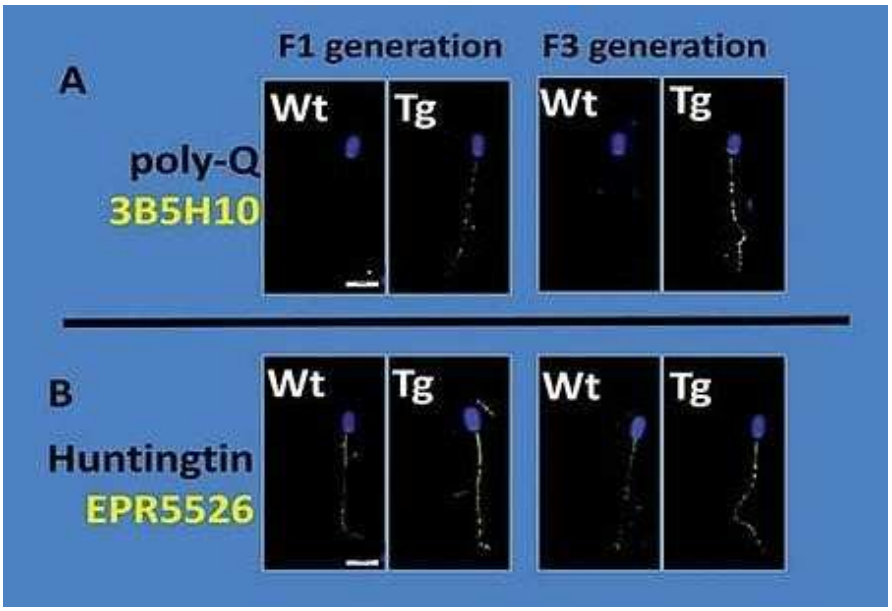
Pomocou ^{31}P MR spektroskopie sme zistili výrazné zníženie pomeru PDE / γ-ATP (p = 0,022) u TgHD kancov v porovnaní s WT súrodencami a žiaden rozdiel v koncentrácii PME a Pi (obr.č. 22).



Obr.č. 22 ***Distribúcia pomerov PDE/γ-ATP, PME/γ-ATP and Pi/γ—ATP u TgHD kancov a ich Wt súrodencov*** *Horizontálne čiary zobrazujú priemerné hodnoty a štandardné odchýlky. Aste-Rix znamenajú významnú úroveň p <0,05.*
(obrázok z publikácie (Jozefovicova et al., 2016))

Imunohistochemické pokusy

Ejakulované spermie kancov boli farbené anti –polyQ protilátkou 3B5H10. U transgénnych spermií z F1 a F3 generácie bol detegovaný fluorescenčný signál pozdĺž celého bičíka spermie, zatiaľ čo u WT súrodencov tento signál nebol pozorovaný. Protilátka EPR5526 proti N-koncovej časti proteínu huntingtínu vykazovala bodkované farbenie v bičíkoch oboch genotypov, avšak fluorescenčný signál bol silnejší u spermií TgHD kancov a vykazoval štatistickú významnosť (obr.č. 23, 24).



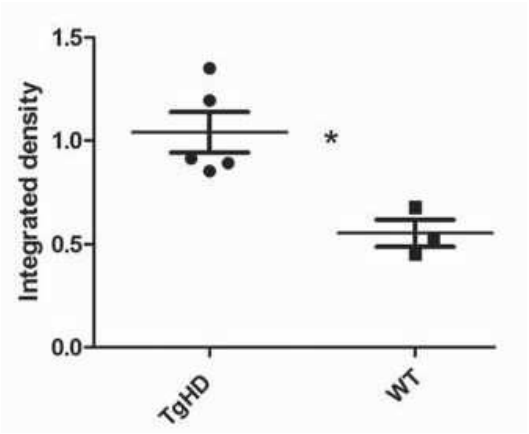
Obr.č. 23 ***Imunofluorescenčné farbenie spermií TgHD a WT kancov z F1 a F3 generácie***

Pomocou protilátky 3B5H10 nebol nájdený žiadny špecifický poly-Q signál v spermiách WT.

Značný pozitívny signál 3B5H10 (žltý)bol pozorovaný výlučne v TgHD spermiách

Anti-huntingtínová špecifická protilátka EPR5526 odhalila huntingtínové epitopy vo WT aj TgHD spermiách obidvoch generácií. Bodové farbenie bolo pri Tg intenzívnejšie ako v prípade spermií WT. Hlavičky spermií boli kontrastne farbené s DAPI (modrá).

(obrázok z publikácie (Jozefovicova et al., 2016))



Obr.č. 24 ***Kvantifikácia florescenčného signálu v spermiáchfarbených EPR5526*** *Meranie bolo uskotočnené v programe ImageJ. Na štatistickú analýzu sa použil Studentov t-test po vyhodnotení normálnej distribúcie pomocou Shapiro-Wilk testu.*

(obrázok z publikácie (Jozefovicova et al., 2016))

Aj keď náš model miniprasaťa pre Huntingtonovu chorobu zatiaľ neprejavuje typické klinické príznaky ako nekontrolované pohyby svalov alebo stratu hmotnosti, tak sa prejavil u tohto modelu výrazný problém s plodnosťou TgHD kancov. Prvotné zmeny v počte spermií, motilite a progresivite sme opísali v publikácii *Mutated huntingtin causes testicular pathology in transgenic minipig boars.* Publikáciou *^{31}P MR Spectroscopy of the Testes and Immunohistochemical Analysis of Sperm of Transgenic Boars Carried N terminal Part of Human Mutated Huntingtin* sme potvrdili ďalšie zmeny na reprodukčnom aparáte TgHD miniprasaťa. ^{31}P MR spektrometria je neinvazívna technika, ktorá môže byť použitá na monitorovanie funkcie semenníkov a dôležité je, že tento článok predstavuje prvú ^{31}P MR spektroskopiu použitú na modely veľkého zvieraťa pre HCH. Na základe vyšetrenia magnetickou rezonanciou bolo zistené signifikantné zníženie relatívnej koncentrácie fosfodiesterov v testikulárnom parenchýme TgHD kancov v porovnaní s netransgennými jedincami rovnakej vekovej kategórie. Taktiež imunohistochemická analýza spermií odhalila výrazné anti-polyQ špecifické (3B5H10) farbenie, ako aj signifikantné zvýšenie antihuntingín farbenie (EPR5526) v bičíkoch TgHD spermií v porovnaní s netransgennými spermiami. Na základe týchto výsledkov môžeme usudzovať, že ľudský mutovaný huntingtín má negatívny vplyv na metabolizmus semenníkov a spôsobuje zvýšený výskyt abnormálnych spermií.

4.3. Mitochondriálny metabolizmus na veľkom zvieracom modely pre Huntingtonovú chorobu: hľadanie biomarkerov v spermiách presymptomatických miniprasiat

Biomarkery mitochondriálneho metabolizmu v spermiách TgHD kancov ...

Mitochondrial Metabolism in Large-Animal Model of Huntington's Disease: The Hunt for Biomarkers in the Spermatozoa of Presymptomatic Minipigs

Running title: Mitochondrial metabolism biomarkers in TgHD boar spermatozoa…

Jana Krizova, Hana Stufkova, Marie Rodinova, Monika Macakova, **Bozena Bohuslavova,** Daniela Vidinska, Jiri Klima, Zdenka Ellederova, Antonin Pavlok, David S. Howland, Jiri Zeman, Jan Motlik and Hana Hansikova

Neuro-Degenerative Diseases, *17*(4–5), 213–226. https://doi.org/10.1159/000475467
IF (2017) 2,842

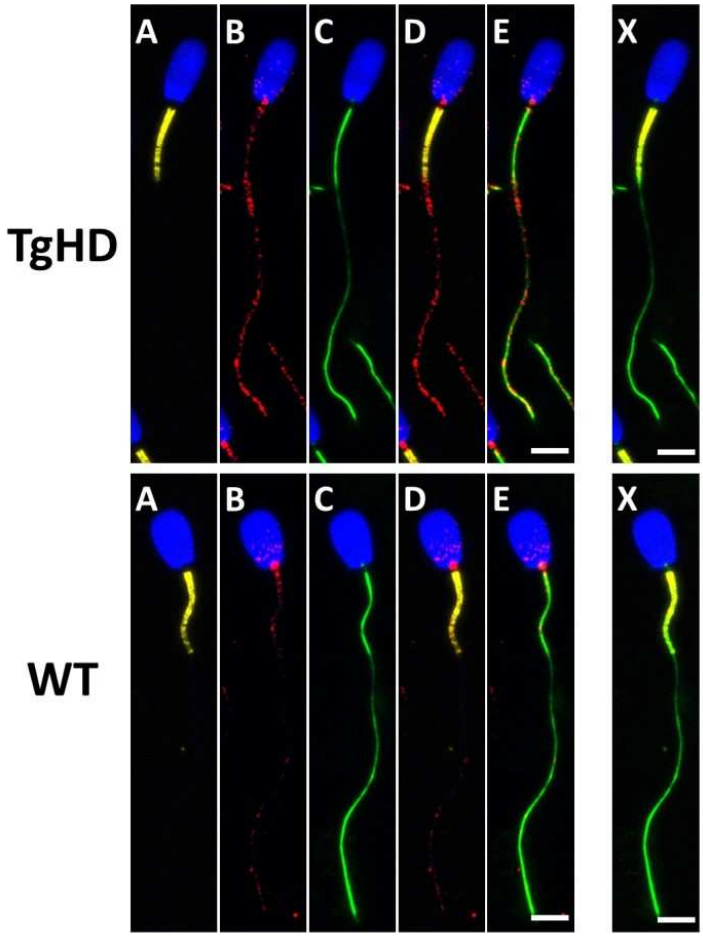
Cieľom štúdie bolo optimalizovať metódy merania mitochondriálneho metabolizmu v spermiách a stanoviť možné biomarkery HCH v spermiách TgHD kancov, ktoré exprimujú N-terminálnu časť mutovaného ľudského huntingtínu. V priebehu štúdie sa opakovane odoberali vzorky 12 TgHD a 12 WT kancov vo veku 12-65 mesiacov. Respiračné parametre mitochondrií boli merané polarografiou, mitochondriálny metabolizmus bol hodnotený detekciou oxidácie rádioaktívne značených substrátov (MEGS) a obsah oxidačných fosforylačných systémových podjednotiek bol detegovaný Western blotom. Štatisticky boli vyhodnotené tri možné faktory, ovplyvňujúce výsledky a to účinok HCH, filiálna generácia a starnutie.

Keďže existuje prebiehajúca diskusia o tom či sa metabolizmus spermií spolieha na energiu s OXPHOS alebo z glykolýzy, eventuálne oboje v štúdii sme sa zamerali na vybrané parametre mitochondriálneho metabolizmu.. Glykolýza sa javí ako kľúčová pre motilitu spermií (Mukai & Okuno, 2004). Avšak kapacitácia, ktorá je dôležitá pre penetráciu cez zónu pellucidu je charakterizovaná hyperaktívnou pohyblivosťou spôsobenou zvýšenou amplitúdou flagelárnych úderov a vyššou produkciou ATP, ktorú vedie OXPHOS v mitochondriách

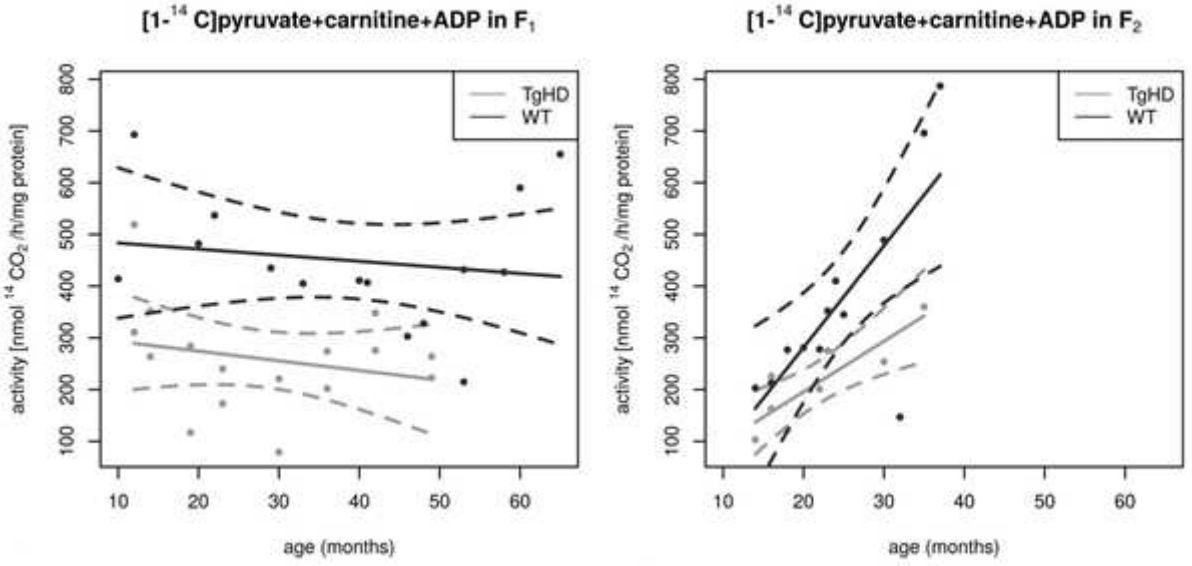
(Stendardi et al., 2011). V prvom rade sme zistili, že polyQ signál mtHtt je lokalizovaný pozdĺž celého bičíka (Macakova et al., 2016) (obr.č. 25). Keďže signál bol viditeľný aj v strednom oddiely bičíka, ktorý je bohatý na mitochondrie, to naznačilo že by mohla nastať priama interakcia mtHtt s mitochondriou. Po optimalizácií meraní mitochondriálneho metablizmu sa štúdia zamerala na identifikáciu biomarkerov MM Huntingtonovej choroby v spermiách TgHD kancov. Pri meraní MEGS (mitochondriálny systém generujúci energiu) sme sa zamerali na štatistickú analýzu 3 možných rušivých faktorov a to účinok samotnej HCH, filiálnu generáciu a starnutie. Analýza odhalila rozdiely medzi TgHD a WT.

Z celkového počtu 36 meraných parametrov 8 súviselo s odlišnou filiálnou generáciou. Tieto parametre boli taktiež ovplyvnené starnutím. Inkubácia 2 ([1-14C] pyruvát + karnitín + ADP) bola znížená u zvierat TgHD a tiež bola významne ovplyvnená vekom a filiálnou generáciou zvieraťa. Inkubácie 4 ([1-14C] pyruvát + ADP) a 6 ([U14C] malát + pyruvát + malonát + ADP), pomer 6 / CS a dýchanie závislé od komplexu I po GMDc-rot (glutamát + malát + ADP + cytochróm c) boli významne znížené u zvierat TgHD (obr.č. 26, 27).

SDS-PAGE imunobloty vybraných podjednotiek OXPHOS a PDHc (pyruvát dehydrogenázový komplex) v spermiách získaných vo veku 47 mesiacov nepreukázali významné poruchy obsahu podjednotky PDHc. V spermiách oboch jedincov TgHD sa zistil len mierne znížený obsah podjednotky SDHA (sukcinát dehydrogenáza A) (SDH70 komplexu II) a akonitázy 2 (obr.č. 28). V prípade SDHA sme pozorovali zníženie množstva podjednotky s vekom u TgHD. Pri porovnaní spermií od tých istých kancov vo veku 14 a 47 mesiacov dosiahlo toto množstvo približne 50% vekovej kontroly. Hoci iba podiely SDHA podjednotky boli nižšie pri Western blotoch, histochemický test odhalil mierny nepomer v aktivitách komplexov OXPHOS medzi vzorkami. Už vo veku 14 mesiacov boli aktivity komplexov II a IV nižšie, zatiaľ čo aktivity komplexov I a V boli vyššie u TgHD v porovnaní s spermiami WT .



Obr.č. 25 ***Lokalizácia mitochondrií a proteínov*** *(proteíny polyQ vrátane mtHtt) v spermiách TgHD kancov WT kancov A) Mitochondriálna časť je zafarbená anti-MTCO1 protilátkou (žltá). B) anti-polyglutamínová časť (PolyQ), klon 3B5H10, protilátka (červená) je významná v spermiách TgHD. C) Mikrotubuly sú farbené anti-acetylovanou tubulínovou (AcTub) protilátkou (zelená). D) zlúčené signály pre mitochondriálny signál MTCO1 a polyQ. E) PolyQ bol lokalizovaný pozdĺž celého koncového konca pozitívneho na AcTb. X) Zlúčené signály pre mitochondriu (anti-MTCO1) a acetylovaný tubulín. Stupnica na mierke predstavuje 5µm. (obrázok z publikácie Krizova et al., 2017)*

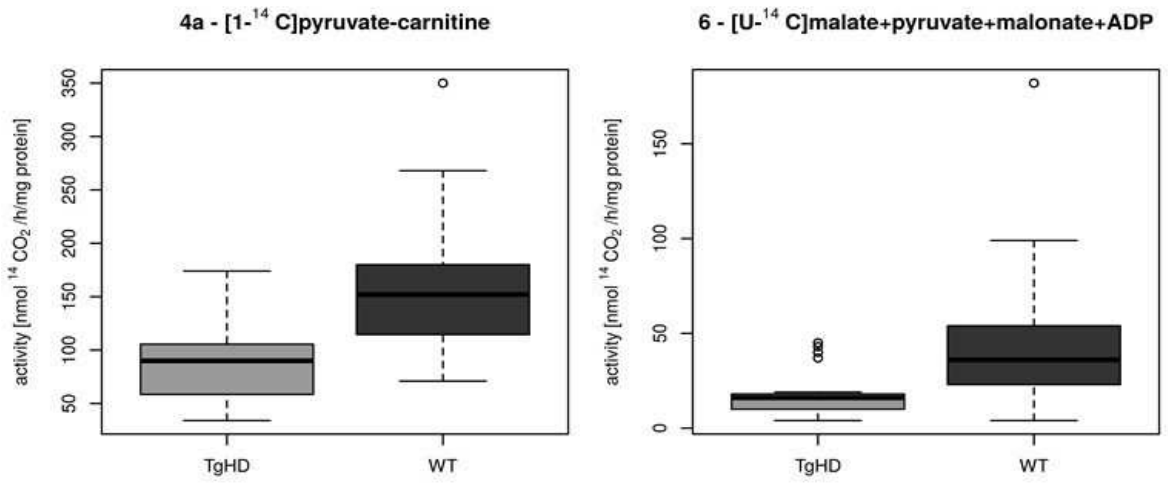


A B

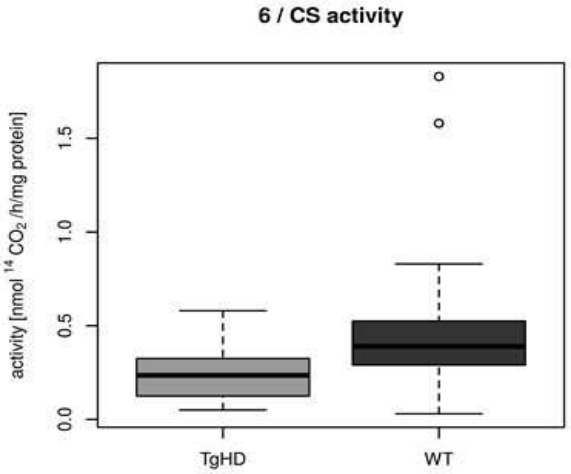
Obr.č. 26 ***Inkubácia MEGS s [1-14C] pyruvátom + karnitínom + ADP***

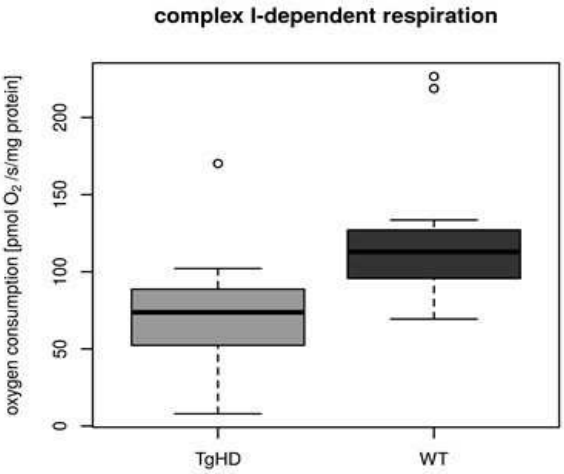
V inkubácii MEGS s [1-14C] pyruvátom + karnitínom + ADP (inkubácia 2) boli všetky rozdiely závislé od HCH, veku a filiálnej generácie významné; P <0,05. A) Inkubácia 2 - aktivita MEGS závisí od [1-14C] pyruvátu + karnitínu + ADP v generácii F1 a B) v F2. V obidvoch generáciách bola účinnosť výrazne nižšia u TgHD kancov (svetlošedá). Pozorovaný parameter však závisel nielen od stavu HCH, ale závisel aj od veku a vykazoval odlišnú postupnosť medzi generáciami (všimnite si opačný sklon lineárnej regresie).

(obrázok z publikácie Krizova et al., 2017)



A B



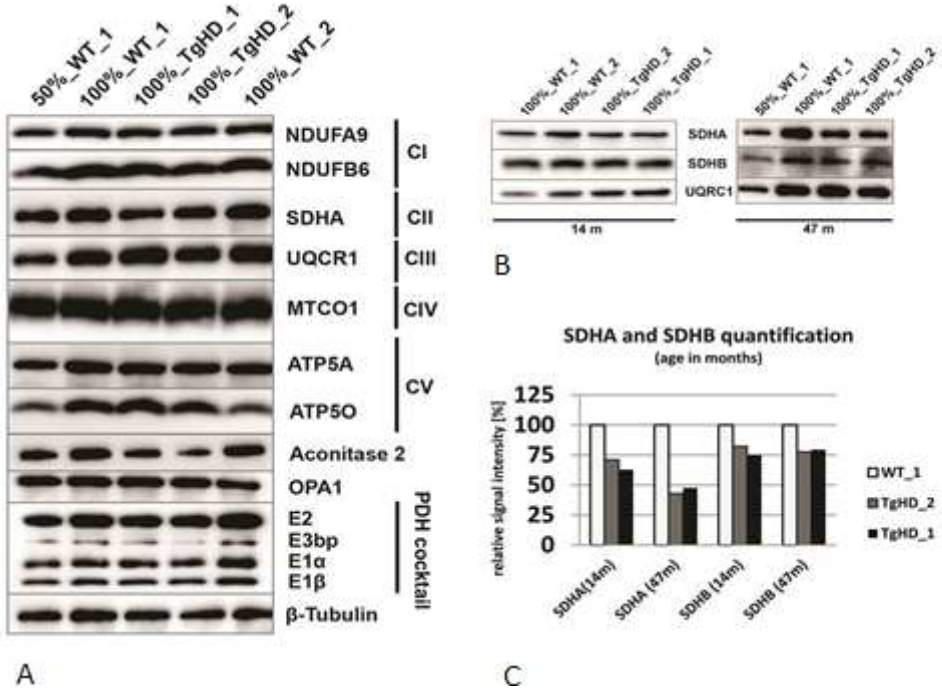


C D

Obr.č. 27 ***Systém mitochondriálnej energie (MEGS) a respiračné parametre****, ktoré preukázali významne nižšie hodnoty u TgHD kancov, a nie sú závislé na filiálnej generácii a veku kancov v čase merania. Grafy (A) a (B): Inkubácie 4a a 6 - MEGS oxidácia v závislosti na [14C] pyruváte (inkubácia 4a) a [U14C] malát + pyruvát + malonát (inkubácia 6) WT (tmavošedá) spermie. Grafy (C) a (D): Inkubácia 6 vyjadrená ako pomer k aktivite citrátovej syntázy (6 / CS) a dýchanie závislé od komplexu I po glutamáte + maláte + ADP + cytochróme c korigované reziduálnym dýchaním nerozvetveným rotenónom) V TgHD (svetlošedá) v porovnaní s spermiami WT (tmavošedá). V súbore vzoriek boli rýchlosti oxidácie v inkubáciách 4a a 6 významne nižšie u spermií TgHD. TgHD spermie tiež vykazovali znížený pomer 6 / CS, ako aj komplexné I závislé dýchanie. Neboli pozorované*

žiadne korelácie s vekom alebo generáciou, čo podporuje použitie týchto parametrov pri ďalšom sledovaní HD u spermií TgHD; P <0,05;

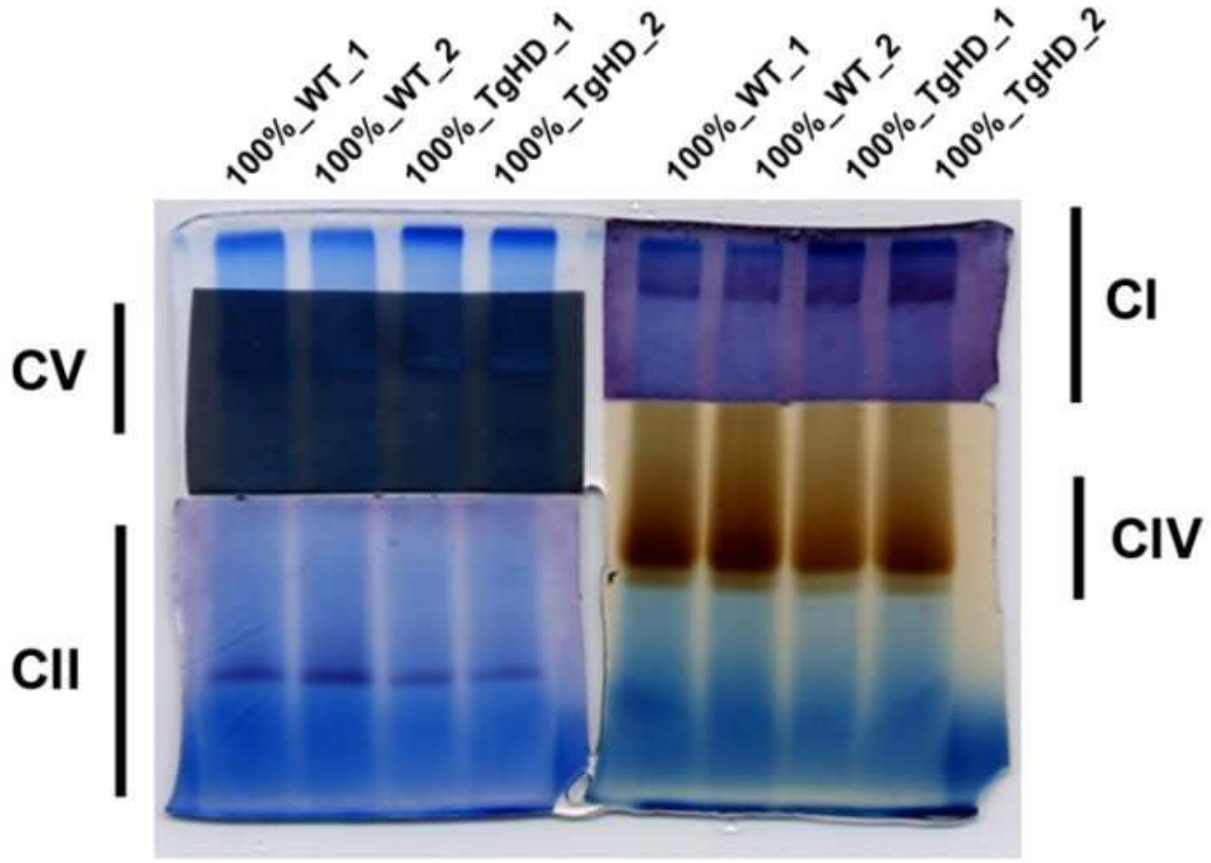
(obrázok z publikácie Krizova et al., 2017)



Obr.č. 28 ***Analýza vybraných podjednotiek oxidačného fosforylačného systému (OXPHOS) a komplexu pyruvátdehydrogenázového komplexu (PDHc) u TgHD kancov***

A) Imunobloty SDS-PAGE vybraných podjednotiek OXPHOS a PDHc získaných spermií Vo veku 47 mesiacov nevykazovali výrazné poruchy obsahu podjednotiek PDHc. Iba mierne znížený obsah podjednotky SDHA (SDH70; CII) a akonitázy 2 sa zistili v spermiách TgHD. (B, C) V závislosti od veku zmeny zložených II podjednotiek . Obsah SDHA a SDHB v spermiách TgHD v porovnaní s WT: SDS-PAGE imunoblot vykazujúci poruchu obsahu SDHA a SDHB podjednotiek v spermiách vo veku 14 (vľavo) a 47 (Vpravo) mesiacov;

(obrázok z publikácie Krizova et al., 2017)



Obr.č. 29 ***Katalytická aktivita oxidačných fosforylačných komplexov (OXPHOS) u spermií TgHDkancov vo veku 14 mesiacov.*** *Analýza bola uskutočnená pomocou histochemických farbiacich metód po oddelení komplexov elektroforézou na báze modrého nativneho polyakrylamidového gélu. Bola pozorovaná mierne znížená aktivita CII a CIV. Neboli zistené žiadne veľké rozdiely v ďalších komplexných aktivitách OXPHOS. CI - NADH: ubichinón oxidoreduktáza; CII-sukcinát dehydrogenáza; CIV - cytochróm c oxidáza; CV - ATP syntáza)*

(obrázok z publikácie Krizova et al., 2017)

V tejto štúdii sme analyzovali mitochondriálny metabolizmus (MM) spermií od TgHD kancov miniprasiat nesúcich N-terminálnu časťľudské mtHtt génu na chromozóme 1. Zmeny v MM môžu byť dôležitým biomarkerom HCH. Na štúdium boli použité metódy, ktoré sa široko uplatňujú v molekulárnej diagnostike mitochondriálnych porúch. To môže pomôcť objasniť etiopatogenézu HCH u TgHD miniprasiat a prehĺbiť vedomosti potrebné pre testovanie nových liekov. V priebehu longitudinálnej štúdie sme identifikovali štyri biomarkery, ktoré naznačujú nešpecifickú patológiu v komplexe I oxidačného fosforylačného systému a v glykolytickom metabolizme, ktorá môže byť prepojená s toxickou funkciou malých fragmentov mtHtt, ktoré sú prítomné v mozgu a semenníkoch u TgHD kancov.

5. DISKUSIA

Mnoho vedeckých tímov na celom svete sa zaoberá vynájdením účinnej liečby na zatiaľ nevyliečiteľné choroby. Na tieto výskumy sa vynakladajú veľké finančné prostriedky. Pre vynájdenie účinnej terapie je potrebné poznať príčinu choroby, ale taktiež poznať fenotyp choroby. Na dôkladné preštudovanie fenotypu sú vytvorené animálne modely chorôb. Tieto modely môžu byť genetické, pri ktorých je vnesený mutovaný gén ochorenia do zvieraťa, alebo negenetické, pri ktorých sa podávajú chemické látky na vyvolanie príznakov ochorenia.

Vo svojej práci sa venujem preštudovaniu fenotypu Huntingtonovej choroby zameraného na testikulárnu degeneráciu. Na štúdium bol použitý model transgénneho miniprasaťa pre HCH (TgHD), ktorý bol vytvorený v roku 2009 pod vedením prof. Motlíka na AV ČR v Liběchove.

Prvým špecifickým cieľom bolo popísať funkčné a štrukturálne zmeny spermií u TgHD kancov. Potvrdili sme expresiu mtHtt v bičíku spermie a v semenníkoch (obr. č. 21, 23), čo by poukazovalo aj na poznatok že najväčšia expresia proteínu Htt prebieha v mozgu a semenníkoch (S. H. Li et al., 1993), a že rozsah expresie v semenníkoch je porovnateľný s expresiou v mozgu (Guo et al., 2003). Sledujeme znížený počet spermií (obr. č. 14,15), zníženú progresivitu (obr. č. 17) a motilitu spermií (obr. č. 14, 16) a nedostatočnú penetračnú schopnosť TgHD spermií oproti WT súrodencov (obr.č.18). Rozdielne hodnoty týchto parametrov sa začali prejavovať vo veku 13 mesiacov a postupom času sa rozdiely prehlbovali. Tieto zmeny taktiež pozoroval Van Raamsdonk u ľudských pacientov, u ktorých bolo robené vyšetrenie reprodukčného aparátu (Van Raamsdonk et al., 2007). Navyše analýza EM odhalila deformáciu mitochondriálneho plášťa v chvostovej časti tkaniva spermií TgHD. Zaznamenali sa tiež zvinuté chvosty a dvojité alebo trojité axonémy (obr. č. 19). To môže byť spôsobené poruchou rozdelenia prebytku cytoplazmy, čo vedie k prítomnosti cytoplazmatických kvapôčok. Tento jav, spolu s dysfunkciou motility spermií, môže súvisieť so znížením mitochondriálneho energetického metabolizmu a funkčného poškodenia komplexu II respiračného reťazca. Občas sa deformácia jadra spája s neúplnou kondenzáciou chromatínu a abnormálny akrozóm sa vyskytol u TgHD spermií, ale nie u WT kontrol. Tieto abnormality boli výraznejšie u F2 generácie. Navyše takmer všetky spermie od TgHD zvierat

z F2 generácie obsahovali cytoplazmatické kvapôčky. Zaznamenaná testikulárna degenerácia je v súlade s pozorovaním u myší (R6 / 2 a Yac72) (Leavitt et al., 2001; Sathasivam et al., 1999). U niektorých TgHD kancov je pozorovaná atrofia semenníkov, čo pozorovala Papalexi na myších modeloch HCH (Papalexi et al., 2005; Van Raamsdonk et al., 2005, 2007). Predchádzajúce štúdie na modeloch HCH hlodavcov ukázali samčiu sterilitu, o ktorej sa predpokladalo, že je dôsledkom zníženia počtu spermií (Sathasivam et al., 1999). Tento fenotyp sme potvrdili vo veľkom zvieracom TgHD miniprasaťa. V histologických vzorkách semenníkov pozorujeme zmenšenie spermatogónii a rozšírenie endoplazmatického retikula. Zníženie počtu vyvíjajúcich sa spermiocytov a spermatíd bolo tiež pozorované u pacientov s HCH (Van Raamsdonk et al., 2007) a YAC128 myší (Van Raamsdonk et al., 2005). Taktiež sme skontrolovali, či vloženie lentivírusového konštruktu neprerušilo žiadnu kódujúcu sekvenciu v genóme ošípaných. Pretože výsledok bol negatívny, otázka bola, či patológia v semenníkoch bola spôsobená mtHtt alebo v dôsledku zmenených hladín hormónov súvisiacich s plodnosťou. Hoci je testikulárna degenerácia v HCH dobre opísaná v modeloch myší (R6 / 2 a YAC128), nie je jasné, či je tento fenotyp nezávislý alebo je dôsledkom zmien hypotalamo-hypofyzárne-gonádovej osi (GnRH). Papalexi opísala významnú stratu neurónov s výrazním znížením GnRH R6/2 myší počnúc vekom 5 týždňov. Po tomto znížení GnRH nasledovalo zníženie hladín testosterónu v plazme vo veku 12 týždňov (Papalexi et al., 2005). Zatiaľ čo atrofia semenníkov bez súbežnej straty GnRH neurónov bola opísaná v modeli myší YAC128 (Van Raamsdonk et al., 2007). Analýza hladín testosterónu u myší YAC128 neodhalila žiadny významný rozdiel v porovnaní s kontrolami, dokonca aj keď už bola prítomná testikulárna atrofia (Van Raamsdonk et al., 2007). Okrem toho Papalexi pri podávaní testosterónu nepozorovala žiadny vplyv na zlepšenie fenotypu HCH pozorovaného u myší R6 / 2, ktoré mali znížený GnRH (Papalexi et al., 2005). Analýza úplného neuroendokrinného statusu u pacientov s HCH meraná Saleh a koloktívom nemala žiadny významný rozdiel v hladinách LH, FSH a testosterónu v plazme medzi všetkými mužskými HCH pacientmi a kontrolami (Saleh et al., 2009). Avšak Markianos pozoroval signifikantne nižšie hladiny testosterónu a LH u pacientov s HCH v porovnaní so zdravými kontrolami (Markianos et al., 2005). Tieto protichodné výsledky sú podobné výsledkom hormonálnej analýzy u nášeho modelu TgHD prasaťa. Nepozorovali sme žiadne významné rozdiely v hladinách hormónov súvisiacich s plodnosťou medzi TgHD a kontrolnými kancami a počas pravidelného odberu spermy neboli pozorované žiadne zmeny libida. Existuje dôkaz, že expresia mutantného Htt vedie k selektívnej bunkovej dysfunkcii a degenerácii (Bhide et

al., 1996). Najviac ovplyvnené bunky sú neuróny. Poskytli sme však aj údaje o degenerácii spermií a testikulárnej dysfunkcii v TgHD modely.

Druhým špecifickým cieľom bolo preštudovanie metabolických porúch v semenníkoch TgHD kancov u F2generácie pomocou ^{31}P MR spektoroskopie. Pretože biopsia je invazívna a môže spôsobiť ďalšie poškodenie spermatogenézy (van der Grond, Laven, Lock, te Velde, & Mali, 1991) tak sme použili ^{31}P MR spektrometriu. Ide o neinvazívnu techniku, ktorá sa môže použiť na monitorovanie funkcie semenníkov, ako je popísané v niekoľkých štúdiách (Srinivas et al., 2002; van der Grond et al., 1992; van der Grond, Van Pelt, et al., 1991; van der Grond, Laven, te Velde, & Mali, 1991). Predchádzajúce ^{31}P MR spektrometrické štúdie na ľudských semenníkoch preukázali, že pomer PME / ATP je citlivejší parameter na monitorovanie funkcie semenníkov, t.j. rozlišovanie medzi normálnymi semenníkmi, oligozoospermiou a azoospermiou ako pomer PDE / ATP alebo Pi / ATP (van der Grond, 1995; van der Grond, Laven, te Velde, et al., 1991). U našich TgHD kancov sme však nezistili významnú zmenu pomeru PME / ATP. Ukázalo sa, že hlavne PDE vrchol pozostáva z glycerofosfocholínu (GPC) a menšieho množstva glycerofosfoetanolamín (GPE) (van der Grond et al., 1992). GPC je prítomný vo veľmi vysokých koncentráciách v semennej plazme a bola zistená pozitívna korelácia medzi koncentráciou GPC a motilitou spermií (Hinton & Setchell, 1980; van der Grond et al., 1992). Preto si myslíme, že znížená hladina pomeru PDE / γ-ATP (obr. č. 22) u TgHD kancov by mohla súvisieť so zníženým množstvom semennej plazmy alebo so zmenami v motilite spermií. Podobné zníženie pomeru PDE / ATP testikulárnych fosfodiesterov (PDE) ($p < 0,05$), ako sme pozorovali u našich TgHD kancov bolo pozorované v štúdii potkanov, ktoré mali počas 10 týždňov podávaný etanol v kvapalnej diéte (Farghali, Williams, Gavaler, & Van Thiel, 1991)

Tretím cieľom bolo preštudovanie mitochondriálnej dysfunkcie spermií kancov miniprasiat transgénnych pre HCH. Keďže v predchádzajúcich štúdiách sme preukázali, že TgHD spermie sú síce schopné naviazať sa na zonu pellucidu, ale majú zníženú schopnosť penetrovať oocyt, čo potom vedie k poklesu plodnosti. Rozhodli sme sa pokúsiť objasniť či je táto patológia spojená s akoukoľvek metabolickou poruchou. Zamerali smesa na podrobnú analýzu mitochondriálneho metabolizmu (MM) TgHD spermií. Zmeny v MM môžu byť dôležitým biomarkerom HCH (Oliveira et al., 2007). Použili sme metódy, ktoré sa široko uplatňujú v molekulárnej diagnostike mitochondriálnych porúch. Najprv sme dokázali, že

mtHtt je prítomný v spermiách a že môže byť v priamom vzájomnom pôsobení so svojimi mitochondriami (obr. č. 25). Pri zameraní sa na vplyv generačného štádia bolo iba 8 z 36 parametrov v analýze ovplyvnených generáciou, ku ktorej patrili zvieratá. Účinok HCH bol preukázaný významným znížením niektorých parametrov kapacity MEGS a dýchania u TgHD kancov.Taktiež vplyv HCH poukázal na päť parametrov užitočných pri monitorovaní progresie HCH a potenciálnych biomarkerov MM HCH u transgénnych kancov (MEGS inkubácie ([1-14C] pyruvát + karnitín + ADP), ([14C] pyruvát + ADP), 6 (U14C) malát + pyruvát + malonát + ADP), pomer 6 / CS a dýchanie závislé od komplexu I (obr. č. 26, 27). Inkubácie, ktoré obsahovali pyruvát, boli znížené, čo vedie k podozreniu, že defekt je spojený s nedostatkom pyruvát dehydrogenázy alebo komplexu I. Na druhej strane zvýšená citlivosť na inhibíciu dýchania závislej od komplexu I bola preukázaná v kostrovom svale myší R6 / 2 (Gizatullina et al., 2006). Pretože inkubácie obsahujúce pyruvát sú významne znížené bez ohľadu na prítomnosť L karnitínu v reakcii, môžeme predpokladať, že metabolický defekt je skôr nezávislý od rýchlosti β-oxidácie mastných kyselín (Brass & Hoppel, 1980). Zistili sme len mierny pokles SDH prostredníctvom histochemického farbenia (obr. č. 29). Predpokladáme, že pozorovaný úbytok obsahu podjednotky SDH a akonitázy je skorá odozva na oxidačný stres pravdepodobne ešte kompenzovaná neznámym mechanizmom počas predsyptomatického štádia HCH. Náš komplexný biochemický prístup k štúdiu spermií TgHD kancov naznačuje možnosť, že malé toxické fragmenty mtHtt môžu interagovať s bunkovým metabolizmom, aby významne zmenili MM spermií a že mitochondriálna dysfunkcia môže spôsobiť narušenú penetračnú aktivitu spermií, prvý predklinický marker HCH u kancov TgHD.

Touto štúdiou sme dokázali, že model TgHD miniprasaťa je vhodným modelom pre štúdium fenotypu HCH.

6. ZÁVER

Študovanie fenotypu u modelov transgénnych zvierat je veľmi dôležité na pochopenie princípov progresie ohorenia a skúšania nových terapeutických princípov na zabránenie ďalšej progresie ochorenia a dáva nádej na vynájdenie liečby vedúcej k úplnému vyliečeniu. Tieto štúdie sú hlavne potrebné u tých ochorení, u ktorých doteraz nie je dostupná žiadna liečba .

Dizertačná práca bola zameraná na štúdium fenotypu pohlavného aparátu TgHD kanca s N-terminálnym koncom pre ľudský mutovaný huntingtín. U nášho modelu doteraz neboli pozorované typické prejavy fenotypu ako mimovoľné pohyby, nekoordinovaná chôdza, ale boli pozorované problémy v reprodukcii. Pri pravidelnom pripúšťaní TgHD kancami sme začali pozorovať, že prasnice nezostávali gravidné. Okrem toho sme začali pozorovať aj iné reprodukčné problémy kancov už vo veku 13 mesiacov (Baxa et al., 2013). Preto sme sa rozhodli dôkladne preštudovať fenotyp reprodukčného aparátu TgHD kancov. Nevedeli sme či to má súvis s HCH, tak sme sa to snažili dokázať.

Aj keď väčšina ľudských pacientov s HCH má potomkov, neznamená to, že by nemali problémy s reprodukciou. Väčšina z nich splodí potomkov pred vypuknutím prvých príznakov bez toho, aby vedeli že túto chorobu majú. Problémy s reprodukciou sa prejavujú až v neskorších štádiách choroby. Náš model miniprasaťa pre HCH vykazuje značné reprodukčné problémy. Je výrazne znížený počet spermií TgHD kancov oproti WT súrodencov, ale okrem zníženia celkového počtu spermií je taktiež výrazne znížená progresivita a motilita. Znížená schopnosť penetrovať oocyt spermiou je najskôr spôsobená poškodením metabolizmom mitochondrií (MM), čím spermie majú nedostatok energie. Poškodenie MM má najskôr súvisí s mtHtt, ktorý bol dokázaný, že sa vyskytuje aj v strednom oddiely bičíka, ktorý je bohatý na mitochondrie. Predpokladá sa vzájomné pôsobenie.

Keďže HCH stále patrí k nevyliečiteľným chorobám, a príznaky choroby sa len utlmujú, ale nelieči sa príčina choroby, ktorá je v dnešnej dobe už známa, vedci na celom svete sa snažia prísť na účinnú liečbu, ktorá by sa mohla použiť na klinickú liečbu. K vynájdeniu liečby, je potrebné dokonalé preštudovanie fenotypu HCH. Touto prácou sme popísali biomarkery HCH, pomocou ktorých by sa dala určiť efektivita liečby.

7. ZOZNAM CITOVANEJ LITERATÚRY

Bär, K. J., Boettger, M. K., Andrich, J., Epplen, J. T., Fischer, F., Cordes, J., … Agelink, M. W. (2008). Cardiovagal modulation upon postural change is altered in Huntington's disease. *European Journal of Neurology*, *15*(8), 869–871. https://doi.org/10.1111/j.1468-1331.2008.02173.x

Baxa, M., Hruska-Plochan, M., Juhas, S., Vodicka, P., Pavlok, A., Juhasova, J., … Motlik, J. (2013). A transgenic minipig model of Huntington's Disease. *Journal of Huntington's Disease*, *2*(1), 47–68. https://doi.org/10.3233/JHD-130001

Bennett, C. F., & Swayze, E. E. (2010). RNA Targeting Therapeutics: Molecular Mechanisms of Antisense Oligonucleotides as a Therapeutic Platform. *Annual Review of Pharmacology and Toxicology*, *50*(1), 259–293. https://doi.org/10.1146/annurev.pharmtox.010909.105654

Bhide, P. G., Day, M., Sapp, E., Schwarz, C., Sheth, A., Kim, J., … DiFiglia, M. (1996). Expression of normal and mutant huntingtin in the developing brain. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience*, *16*(17), 5523–5535. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/8757264

Bjarkam, C. R., Jorgensen, R. L., Jensen, K. N., Sunde, N. A., & Sørensen, J.-C. H. (2008). Deep brain stimulation electrode anchoring using BioGlue®, a protective electrode covering, and a titanium microplate. *Journal of Neuroscience Methods*, *168*(1), 151–155. https://doi.org/10.1016/j.jneumeth.2007.09.008

Bohuslavová B., Mačáková M., (2015), Testikulárna degenerácia pri huntingtonovej chorobe *Bioprospect, 25th Volume, No. 1/2015,8-10.*

Brass, E. P., & Hoppel, C. L. (1980). Relationship between acid-soluble carnitine and coenzyme A pools in vivo. *The Biochemical Journal*, *190*(3), 495–504. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/7470064

Busse, M. E., Hughes, G., Wiles, C. M., & Rosser, A. E. (2008). Use of hand-held dynamometry in the evaluation of lower limb muscle strength in people with Huntington's disease. *Journal of Neurology*, *255*(10), 1534–1540. https://doi.org/10.1007/s00415-008-0964-x

Cattaneo, E., Rigamonti, D., Goffredo, D., Zuccato, C., Squitieri, F., & Sipione, S. (2001). Loss of normal huntingtin function: new developments in Huntington's disease research. *Trends in Neurosciences*, *24*(3), 182–188. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/11182459

Cattaneo, E., Zuccato, C., & Tartari, M. (2005). Normal huntingtin function: an alternative approach to Huntington's disease. *Nature Reviews. Neuroscience*, *6*(12), 919–930. https://doi.org/10.1038/nrn1806

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. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/10700648

Ciammola, A., Sassone, J., Alberti, L., Meola, G., Mancinelli, E., Russo, M. A., … Silani, V. (2006). Increased apoptosis, Huntingtin inclusions and altered differentiation in muscle cell cultures from Huntington's disease subjects. *Cell Death and Differentiation*, *13*(12), 2068–2078. https://doi.org/10.1038/sj.cdd.4401967

Copyright © 2004 Pearson Education, Inc., publishing as Benjamin Cummings Fundamentals of Anatomy & Physiology SIXTH EDITION Frederic H. Martini PowerPoint. - ppt download. (n.d.). Retrieved February 21, 2018, from http://slideplayer.com/slide/8703957/

Cornett, J., Cao, F., Wang, C.-E., Ross, C. A., Bates, G. P., Li, S.-H., & Li, X.-J. (2005). Polyglutamine expansion of huntingtin impairs its nuclear export. *Nature Genetics*, *37*(2), 198–204. https://doi.org/10.1038/ng1503

Costabile, R. A., & Spevak, M. (2001). Characterization of patients presenting with male

factor infertility in an equal access, no cost medical system. *Urology*, *58*(6), 1021–1024. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/11744480

Čeřovský J., 2001b: Inseminace prasat, s. 106–134. In: Louda F. (ed.), *Inseminace hospodářských zvířat se základy biotechnických metod*. ČZU, Praha, 225 s., ISBN 80-2130702-1.

de Die-Smulders, C. E. M., de Wert, G. M. W. R., Liebaers, I., Tibben, A., & Evers-Kiebooms, G. (2013). Reproductive options for prospective parents in families with Huntington's disease: clinical, psychological and ethical reflections. *Human Reproduction Update*, *19*(3), 304–315. https://doi.org/10.1093/humupd/dms058

Dietrich, P., Shanmugasundaram, R., E, S., & Dragatsis, I. (2008). Congenital hydrocephalus associated with abnormal subcommissural organ in mice lacking huntingtin in Wnt1 cell lineages. *Human Molecular Genetics*, *18*(1), 142–150. https://doi.org/10.1093/hmg/ddn324

Djoussé, L., Knowlton, B., Cupples, L. A., Marder, K., Shoulson, I., & Myers, R. H. (2002). Weight loss in early stage of Huntington's disease. *Neurology*, *59*(9), 1325–1330. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/12427878

Dowhan David (June 1, 2013). [Retrieved

fromhttp://web.b.ebscohost.com/ehost/pdfviewer/pdfviewer?sid=f98b06cd-8924-4f6d-89f8245bde9615f8%40sessionmgr198&vid=2&hid=124

Duff, K., Paulsen, J. S., Beglinger, L. J., Langbehn, D. R., Wang, C., Stout, J. C., … Group, P.-H. I. of the H. (2010). "Frontal" Behaviors Before the Diagnosis of Huntington's Disease and Their Relationship to Markers of Disease Progression: Evidence of Early Lack of Awareness. *The Journal of Neuropsychiatry and Clinical Neurosciences*, *22*(2), 196–207. https://doi.org/10.1176/jnp.2010.22.2.196

Dusenbery, D. B. (2009). *Living at micro scale : the unexpected physics of being small*.

Harvard University Press.

Duyao, M. P., Auerbach, A. B., Ryan, A., Persichetti, F., Barnes, G. T., McNeil, S. M., … Joyner, A. L. (1995). Inactivation of the mouse Huntington's disease gene homolog Hdh. *Science (New York, N.Y.)*, *269*(5222), 407–410. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/7618107

Edirisinghe, W. R., Murch, A. R., Junk, S. M., & Yovich, J. L. (1998). Cytogenetic analysis of unfertilized oocytes following intracytoplasmic sperm injection using spermatozoa from a globozoospermic man. *Human Reproduction (Oxford, England)*, *13*(11), 3094–3098. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/9853863

EDDY E. M., 2006: The spermatozoon, s. 3–54. In: Neill J. (ed.), *Knobil and Neill's Physiology of Reproduction*, *Third Edition*. Elsevier, San Diego, 3230 s., ISBN 978-0-12515400-0.

Escalier, D. (2003). New Insights into the Assembly of the Periaxonemal Structures in Mammalian Spermatozoa. *Biology of Reproduction*, *69*(2), 373–378. https://doi.org/10.1095/biolreprod.103.015719

Farghali, H., Williams, D. S., Gavaler, J., & Van Thiel, D. H. (1991). Effect of short-term ethanol feeding on rat testes as assessed by 31P NMR spectroscopy, 1H NMR imaging, and biochemical methods. *Alcoholism, Clinical and Experimental Research*, *15*(6), 1018–1023. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/1789376

Flörke, S., Phi-van, L., Müller-Esterl, W., Scheuber, H. P., & Engel, W. (1983). Acrosin in the spermiohistogenesis of mammals. *Differentiation; Research in Biological Diversity*, *24*(3), 250–256. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/6354818

Gamčík P., Kozumplík J., Schwarc F., Vlček Z., Zibrín M., 1976: *Umelá inseminácia a andrologia hospodárskych zvierat*. Príroda, Bratislava, 574 s., ISBN 64-025-76.

Gauthier, L. R., Charrin, B. C., Borrell-Pagès, M., Dompierre, J. P., Rangone, H., Cordelières,

F. P., … Saudou, F. (2004). Huntingtin Controls Neurotrophic Support and Survival of Neurons by Enhancing BDNF Vesicular Transport along Microtubules. *Cell*, *118*(1), 127–138. https://doi.org/10.1016/j.cell.2004.06.018

Gizatullina, Z. Z., Lindenberg, K. S., Harjes, P., Chen, Y., Kosinski, C. M., Landwehrmeyer, B. G., … Gellerich, F. N. (2006). Low stability of huntington muscle Mitochondria against Ca2+ in R6/2 mice. *Annals of Neurology*, *59*(2), 407–411. https://doi.org/10.1002/ana.20754

Goodman, A. O. G., Murgatroyd, P. R., Medina-Gomez, G., Wood, N. I., Finer, N., Vidal-Puig, A. J., … Barker, R. A. (2008). The metabolic profile of early Huntington's disease-a combined human and transgenic mouse study. *Experimental Neurology*, *210*(2), 691–698. https://doi.org/10.1016/j.expneurol.2007.12.026

Guo, J., Zhu, P., Wu, C., Yu, L., Zhao, S., & Gu, X. (2003). In silico analysis indicates a similar gene expression pattern between human brain and testis. *Cytogenetic and Genome Research*, *103*(1–2), 58–62. https://doi.org/10.1159/000076290

Gusella, J. F., Persichetti, F., & MacDonald, M. E. (1997). The genetic defect causing Huntington's disease: repeated in other contexts? *Molecular Medicine (Cambridge, Mass.)*, *3*(4), 238–246. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/9131586

Gusella, J. F., Wexler, N. S., Conneally, P. M., Naylor, S. L., Anderson, M. A., Tanzi, R. E., … Martin, J. B. (1983). A polymorphic DNA marker genetically linked to Huntington's disease. *Nature*, *306*(5940), 234–238. https://doi.org/10.1038/306234a0

Halliday, G. M., McRitchie, D. A., Macdonald, V., Double, K. L., Trent, R. J., & McCusker, E. (1998). Regional Specificity of Brain Atrophy in Huntington's Disease. *Experimental Neurology*, *154*(2), 663–672. https://doi.org/10.1006/exnr.1998.6919

Hinton, B. T., & Setchell, B. P. (1980). Concentrations of glycerophosphocholine, phosphocholine and free inorganic phosphate in the luminal fluid of the rat testis and epididymis. *Journal of Reproduction and Fertility*, *58*(2), 401–406. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/7431273

Huntington, G. (2003). On Chorea. *The Journal of Neuropsychiatry and Clinical Neurosciences*, *15*(1), 109–112. https://doi.org/10.1176/jnp.15.1.109

Chan, A. W. S., & Agca, Y. (2008). Transgenic Animal Models of Neurodegenerative Diseases. In *Sourcebook of Models for Biomedical Research* (pp. 323–331). Totowa, NJ: Humana Press. https://doi.org/10.1007/978-1-59745-285-4_35

Jason, G. W., Suchowersky, O., Pajurkova, E. M., Graham, L., Klimek, M. L., Garber, A. T., & Poirier-Heine, D. (1997). Cognitive manifestations of Huntington disease in relation to genetic structure and clinical onset. *Archives of Neurology*, *54*(9), 1081–1088. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/9311351

Kalchman, M. A., Graham, R. K., Xia, G., Koide, H. B., Hodgson, J. G., Graham, K. C., … Hayden, M. R. (1996). Huntingtin is ubiquitinated and interacts with a specific ubiquitin-conjugating enzyme. *The Journal of Biological Chemistry*, *271*(32), 19385–19394. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/8702625

Karban, R. (2008). Plant behaviour and communication. *Ecology Letters*, *11*(7), 727–739. https://doi.org/10.1111/j.1461-0248.2008.01183.x

Keiser, M. S., Kordasiewicz, H. B., & McBride, J. L. (2016). Gene suppression strategies for dominantly inherited neurodegenerative diseases: lessons from Huntington's disease and spinocerebellar ataxia. *Human Molecular Genetics*, *25*(R1), R53–R64. https://doi.org/10.1093/hmg/ddv442

Kim, M. W., Chelliah, Y., Kim, S. W., Otwinowski, Z., & Bezprozvanny, I. (2009). Secondary Structure of Huntingtin Amino-Terminal Region. *Structure*, *17*(9), 1205–1212. https://doi.org/10.1016/j.str.2009.08.002

Kiriazis, H., Jennings, N. L., Davern, P., Lambert, G., Su, Y., Pang, T., … Du, X.-J. (2012). Neurocardiac dysregulation and neurogenic arrhythmias in a transgenic mouse model of Huntington's disease. *The Journal of Physiology*, *590*(22), 5845–5860. https://doi.org/10.1113/jphysiol.2012.238113

Klöppel, S., Stonnington, C. M., Petrovic, P., Mobbs, D., Tüscher, O., Craufurd, D., … Frackowiak, R. S. J. (2010). Irritability in pre-clinical Huntington's disease. *Neuropsychologia*, *48*(2), 549–557. https://doi.org/10.1016/j.neuropsychologia.2009.10.016

Kolárová, J., Vrtěl, R., Vodicka, R., Capková, P., & Santavý, J. (2003). [Microdeletion of the azoospermia factor as one of the causes of male infertility]. *Casopis Lekaru Ceskych*, *142*(4), 211–215. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/12841122

Kotrcova, E., Jarkovska, K., Valekova, I., Zizkova, M., Motlik, J., Gadher, S. J., & Kovarova, H. (2015). Challenges of Huntington's disease and quest for therapeutic biomarkers. *PROTEOMICS - Clinical Applications*, *9*(1–2), 147–158. https://doi.org/10.1002/prca.201400073

Krizova, J., Stufkova, H., Rodinova, M., Macakova, M., Bohuslavova, B., Vidinska, D., … Hansikova, H. (2017). Mitochondrial Metabolism in a Large-Animal Model of Huntington Disease: The Hunt for Biomarkers in the Spermatozoa of Presymptomatic Minipigs. *Neuro-Degenerative Diseases*, *17*(4–5), 213–226. https://doi.org/10.1159/000475467

Kudláč E., 2003: Rozmnoţování (Reprodukce), s. 301–330. In: Jelínek P., Koudela K. (eds.), Fyziologie hospodářských zvířat. MZLU, Brno, 414 s., ISBN 80-7157-644-1.

Leavitt, B. R., Guttman, J. A., Hodgson, J. G., Kimel, G. H., Singaraja, R., Vogl, A. W., & Hayden, M. R. (2001). Wild-Type Huntingtin Reduces the Cellular Toxicity of Mutant Huntingtin In Vivo. *The American Journal of Human Genetics*, *68*(2), 313–324. https://doi.org/10.1086/318207

Levitis, D. A., Lidicker, W. Z., Freund, G., & Freund, G. (2009). Behavioural biologists don't agree on what constitutes behaviour. *Animal Behaviour*, *78*(1), 103–110. https://doi.org/10.1016/j.anbehav.2009.03.018

Li, S. H., Schilling, G., Young, W. S., Li, X. J., Margolis, R. L., Stine, O. C., … Ranen, N. G. (1993). Huntington's disease gene (IT15) is widely expressed in human and rat tissues. *Neuron*, *11*(5), 985–993. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/8240819

Li, X.-J., & Li, S. (2012). Influence of Species Differences on the Neuropathology of Transgenic Huntington's Disease Animal Models. *Journal of Genetics and Genomics*, *39*(6), 239–245. https://doi.org/10.1016/j.jgg.2012.05.002

Lind, N. M., Vinther, M., Hemmingsen, R. P., & Hansen, A. K. (2005). Validation of a digital video tracking system for recording pig locomotor behaviour. *Journal of Neuroscience Methods*, *143*(2), 123–132. https://doi.org/10.1016/j.jneumeth.2004.09.019

Lipe, H., & Bird, T. (2009). Late onset Huntington Disease: clinical and genetic characteristics of 34 cases. *Journal of the Neurological Sciences*, *276*(1–2), 159–162. https://doi.org/10.1016/j.jns.2008.09.029

Macakova, M., Bohuslavova, B., Vochozkova, P., Pavlok, A., Sedlackova, M., Vidinska, D., … Motlik, J. (2016). Mutated Huntingtin Causes Testicular Pathology in Transgenic Minipig Boars. *Neurodegenerative Diseases*, *16*(3–4), 245–259. https://doi.org/10.1159/000443665

Machev, N., Gosset, P., & Viville, S. (2005). Chromosome abnormalities in sperm from infertile men with normal somatic karyotypes: teratozoospermia. *Cytogenetic and Genome Research*, *111*(3–4), 352–357. https://doi.org/10.1159/000086910

Markianos, M., Panas, M., Kalfakis, N., & Vassilopoulos, D. (2005). Plasma testosterone in male patients with Huntington's disease: Relations to severity of illness and dementia. *Annals of Neurology*, *57*(4), 520–525. https://doi.org/10.1002/ana.20428

Marvan F. a kol., 1992: *Morfologie hospodářských zvířat*. ZN Brázda, Praha, 304 s., ISBN 80-209-0226-0.

Meng, X., Lindahl, M., Hyvönen, M. E., Parvinen, M., de Rooij, D. G., Hess, M. W., … Sariola, H. (2000). Regulation of cell fate decision of undifferentiated spermatogonia by GDNF. *Science (New York, N.Y.)*, *287*(5457), 1489–1493. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/10688798

Menzies, F. M., Shepherd, M. C., Nibbs, R. J., & Nelson, S. M. (2011). The role of mast cells and their mediators in reproduction, pregnancy and labour. *Human Reproduction Update*, *17*(3), 383–396. https://doi.org/10.1093/humupd/dmq053

Mihm, M. J., Amann, D. M., Schanbacher, B. L., Altschuld, R. A., Bauer, J. A., & Hoyt, K. R. (2007). Cardiac dysfunction in the R6/2 mouse model of Huntington's disease. *Neurobiology of Disease*, *25*(2), 297–308. https://doi.org/10.1016/j.nbd.2006.09.016

Minton, E. A., & Kahle, L. R. (n.d.). *Belief systems, religion, and behavioral economics : marketing in multicultural environments*. Retrieved from https://www.amazon.com/Belief-Systems-Religion-Behavioral-Economics/dp/1606497049

Mitchell, V., Rives, N., Albert, M., Peers, M.-C., Selva, J., Clavier, B., … Escalier, D. (2006). Outcome of ICSI with ejaculated spermatozoa in a series of men with distinct ultrastructural flagellar abnormalities. *Human Reproduction*, *21*(8), 2065–2074. https://doi.org/10.1093/humrep/del130

Montoya, A., Price, B. H., Menear, M., & Lepage, M. (2006). Brain imaging and cognitive dysfunctions in Huntington's disease. *Journal of Psychiatry & Neuroscience : JPN*, *31*(1), 21–29. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/16496032

Moretti, E., Pascarelli, N. A., Federico, M. G., Renieri, T., & Collodel, G. (2008). Abnormal elongation of midpiece, absence of axoneme and outer dense fibers at principal piece level, supernumerary microtubules: a sperm defect of possible genetic origin? *Fertility and Sterility*, *90*(4), 1201.e3-1201.e8. https://doi.org/10.1016/j.fertnstert.2007.11.050

Morton, A. J., & Howland, D. S. (2013). Large genetic animal models of Huntington's Disease. *Journal of Huntington's Disease*, *2*(1), 3–19. https://doi.org/10.3233/JHD-

130050

Mukai, C., & Okuno, M. (2004). Glycolysis Plays a Major Role for Adenosine Triphosphate Supplementation in Mouse Sperm Flagellar Movement. *Biology of Reproduction*, *71*(2), 540–547. https://doi.org/10.1095/biolreprod.103.026054

Nasir, J., Floresco, S. B., O'Kusky, J. R., Diewert, V. M., Richman, J. M., Zeisler, J., … Hayden, M. R. (1995). Targeted disruption of the Huntington's disease gene results in embryonic lethality and behavioral and morphological changes in heterozygotes. *Cell*, *81*(5), 811–823. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/7774020

Neylan, T. C. (2003). Neurodegenerative Disorders. *The Journal of Neuropsychiatry and Clinical Neurosciences*, *15*(1), 108–108. https://doi.org/10.1176/jnp.15.1.108

O'Donnell, L., Nicholls, P. K., O'Bryan, M. K., McLachlan, R. I., & Stanton, P. G. (2011). Spermiation. *Spermatogenesis*, *1*(1), 14–35. https://doi.org/10.4161/spmg.1.1.14525

Oliveira, J. M. A., Jekabsons, M. B., Chen, S., Lin, A., Rego, A. C., Gonçalves, J., … Nicholls, D. G. (2007). Mitochondrial dysfunction in Huntington's disease: the bioenergetics of isolated and in situ mitochondria from transgenic mice. *Journal of Neurochemistry*, *101*(1), 241–249. https://doi.org/10.1111/j.1471-4159.2006.04361.x

Papalexi, E., Persson, A., Björkqvist, M., Petersén, Å., Woodman, B., Bates, G. P., … Popovic, N. (2005). Reduction of GnRH and infertility in the R6/2 mouse model of Huntington's disease. *European Journal of Neuroscience*, *22*(6), 1541–1546. https://doi.org/10.1111/j.1460-9568.2005.04324.x

Paulsen, J. S., Hoth, K. F., Nehl, C., & Stierman, L. (2005). Critical Periods of Suicide Risk in Huntington's Disease. *American Journal of Psychiatry*, *162*(4), 725–731. https://doi.org/10.1176/appi.ajp.162.4.725

Paulsen, J. S., Ready, R. E., Hamilton, J. M., Mega, M. S., & Cummings, J. L. (2001). Neuropsychiatric aspects of Huntington's disease. *Journal of Neurology, Neurosurgery, and Psychiatry*, *71*(3), 310–314. https://doi.org/10.1136/JNNP.71.3.310

Ramaswamy, S., McBride, J. L., & Kordower, J. H. (2007). Animal models of Huntington's disease. *ILAR Journal*, *48*(4), 356–373. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/17712222

Ramos, E. M., Latourelle, J. C., Gillis, T., Mysore, J. S., Squitieri, F., Di Pardo, A., … MacDonald, M. E. (2013). Candidate glutamatergic and dopaminergic pathway gene variants do not influence Huntington's disease motor onset. *Neurogenetics*, *14*(3–4), 173–179. https://doi.org/10.1007/s10048-013-0364-y

Rasmussen, A., Macias, R., Yescas, P., Ochoa, A., Davila, G., & Alonso, E. (2000). Huntington Disease in Children: Genotype-Phenotype Correlation. *Neuropediatrics*, *31*(4), 190–194. https://doi.org/10.1055/s-2000-7461

Ribaï, P., Nguyen, K., Hahn-Barma, V., Gourfinkel-An, I., Vidailhet, M., Legout, A., … Dürr, A. (2007). Psychiatric and Cognitive Difficulties as Indicators of Juvenile Huntington Disease Onset in 29 Patients. *Archives of Neurology*, *64*(6), 813. https://doi.org/10.1001/archneur.64.6.813

Ribchester, R. R., Thomson, D., Wood, N. I., Hinks, T., Gillingwater, T. H., Wishart, T. M., … Morton, A. J. (2004). Progressive abnormalities in skeletal muscle and neuromuscular junctions of transgenic mice expressing the Huntington's disease mutation. *European Journal of Neuroscience*, *20*(11), 3092–3114. https://doi.org/10.1111/j.1460-9568.2004.03783.x

Rigamonti, D., Bauer, J. H., De-Fraja, C., Conti, L., Sipione, S., Sciorati, C., … Cattaneo, E. (2000). Wild-type huntingtin protects from apoptosis upstream of caspase-3. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience*, *20*(10), 3705–3713. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/10804212

Rockabrand, E., Slepko, N., Pantalone, A., Nukala, V. N., Kazantsev, A., Marsh, J. L., … Thompson, L. M. (2007). The first 17 amino acids of Huntingtin modulate its sub-cellular localization, aggregation and effects on calcium homeostasis. *Human Molecular Genetics*, *16*(1), 61–77. https://doi.org/10.1093/hmg/ddl440

Rosenblatt, A. (2007). Neuropsychiatry of Huntington's disease. *Dialogues in Clinical Neuroscience*, *9*(2), 191–197. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/17726917

Ross C.A., Margolis RL. Huntington disease. In: Davis KL, Chamey D, Coyle JT, Nemeroff C (eds).(2002) Neuropsychopharmacology: The Fifth Generation of Progress. *1st ed. Philadelphia: Lippincott Williams Wilkins*

Ross, C. A., & Tabrizi, S. J. (2011). Huntington's disease: from molecular pathogenesis to clinical treatment. *The Lancet Neurology*, *10*(1), 83–98. https://doi.org/10.1016/S1474-4422(10)70245-3

Roth,J., Klempíř, J., Uhrová, T., (2009) Současné terapeutické možnosti uHuntingtonovy nemoci. *Psychiat. pro praxi 10(5) 205-208*

Roth. (2010) Huntington´s disease. *Cesk Slov Neuro N*. 73/106 (2): 107 – 123.

Roth (2012) Juvenilní forma Huntingtonovy nemoci, *Neurol. prax* 13(3): 133–136

Rowe, P. J., & World Health Organization. (2000). *WHO manual for the standardized investigation, diagnosis, and management of the infertile male*. Published on behalf of the World Health Organization by Cambridge University Press. Retrieved from https://books.google.cz/books/about/WHO_Manual_for_the_Standardized_Investig.html?id=9HUcx4ViQ9kC&redir_esc=y

Rüb, U., Hoche, F., Brunt, E. R., Heinsen, H., Seidel, K., Del Turco, D., … den Dunnen, W. F. (2013). Degeneration of the Cerebellum in Huntington's Disease (HD): Possible Relevance for the Clinical Picture and Potential Gateway to Pathological Mechanisms of the Disease Process. *Brain Pathology*, *23*(2), 165–177. https://doi.org/10.1111/j.1750-

3639.2012.00629.x

Rubinsztein, D. C., Leggo, J., Coles, R., Almqvist, E., Biancalana, V., Cassiman, J. J., … Hayden, M. R. (1996). Phenotypic characterization of individuals with 30-40 CAG repeats in the Huntington disease (HD) gene reveals HD cases with 36 repeats and apparently normal elderly individuals with 36-39 repeats. *American Journal of Human Genetics*, *59*(1), 16–22. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/8659522

Rybouchkin, A., Dozortsev, D., Pelinck, M. J., De Sutter, P., & Dhont, M. (1996). Andrology: Analysis of the oocyte activating capacity and chromosomal complement of round-headed human spermatozoa by their injection into mouse oocytes. *Human Reproduction*, *11*(10), 2170–2175. https://doi.org/10.1093/oxfordjournals.humrep.a019071

ŘÍHA J., Petelíková J., Čeřovský J., Baţant J., Bochenek M.,Pytloun J., 2003: Plemenitba hospodářských zvířat. *VÚCHS, Rapotín, 151 s., ISBN 80-903143-4-1*

Sachdev, P. S. (n.d.). Huntington's Disease and related disorders and their association with schizophrenia-like psychosis. In P. S. Sachdev & M. S. Keshavan (Eds.), *Secondary Schizophrenia* (pp. 348–357). Cambridge: Cambridge University Press. https://doi.org/10.1017/CBO9780511789977.029

Saleh, N., Moutereau, S., Durr, A., Krystkowiak, P., Azulay, J.-P., Tranchant, C., … Maison, P. (2009). Neuroendocrine Disturbances in Huntington's Disease. *PLoS ONE*, *4*(3), e4962. https://doi.org/10.1371/journal.pone.0004962

Saravia, F., Wallgren, M., Johannisson, A., Calvete, J. J., Sanz, L., Peña, F. J., … Rodríguez-Martínez, H. (2009). Exposure to the seminal plasma of different portions of the boar ejaculate modulates the survival of spermatozoa cryopreserved in MiniFlatPacks. *Theriogenology*, *71*(4), 662–675. https://doi.org/10.1016/j.theriogenology.2008.09.037

Sathasivam, K., Hobbs, C., Turmaine, M., Mangiarini, L., Mahal, A., Bertaux, F., … Bates, G. P. (1999). Formation of polyglutamine inclusions in non-CNS tissue. *Human Molecular Genetics*, *8*(5), 813–822. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/10196370

Schwarcz, R., Foster, A. C., French, E. D., Whetsell, W. O., & Köhler, C. (1984). Excitotoxic models for neurodegenerative disorders. *Life Sciences*, *35*(1), 19–32. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/6234446

Siesling, S., Vegter-van der Vlis, M., & Roos, R. A. (1997). Juvenile Huntington disease in the Netherlands. *Pediatric Neurology*, *17*(1), 37–43. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/9308974

Snowden, J., Craufurd, D., Griffiths, H., Thompson, J., & Neary, D. (2001). Longitudinal evaluation of cognitive disorder in Huntington's disease. *Journal of the International Neuropsychological Society : JINS*, *7*(1), 33–44. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/11253840

Srinivas, M., Degaonkar, M., Chandrasekharam, V. V. S. S., Gupta, D. K., Hemal, A. K., Shariff, A., & Jagannathan, N. R. (2002). Potential of MRI and 31P MRS in the evaluation of experimental testicular trauma. *Urology*, *59*(6), 969–972. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/12031396

Steffan, J. S., Agrawal, N., Pallos, J., Rockabrand, E., Trotman, L. C., Slepko, N., … Marsh, J. L. (2004). SUMO Modification of Huntingtin and Huntington's Disease Pathology. *Science*, *304*(5667), 100–104. https://doi.org/10.1126/science.1092194

Stendardi, A., Focarelli, R., Piomboni, P., Palumberi, D., Serafini, F., Ferramosca, A., & Zara, V. (2011). Evaluation of mitochondrial respiratory efficiency during in vitro capacitation of human spermatozoa. *International Journal of Andrology*, *34*(3), 247–255. https://doi.org/10.1111/j.1365-2605.2010.01078.x

Stiles, D. K., Zhang, Z., Ge, P., Nelson, B., Grondin, R., Ai, Y., … Sah, D. W. Y. (2012). Widespread suppression of huntingtin with convection-enhanced delivery of siRNA. *Experimental Neurology*, *233*(1), 463–471. https://doi.org/10.1016/j.expneurol.2011.11.020

Strzeżek, J., Wysocki, P., Kordan, W., Kuklińska, M., Mogielnicka, M., Soliwoda, D., &

Fraser, L. (2005). Proteomics of boar seminal plasma - current studies and possibility of their application in biotechnology of animal reproduction. *Reproductive Biology*, *5*(3), 279–290. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/16372045

Thompson, L. M., Aiken, C. T., Kaltenbach, L. S., Agrawal, N., Illes, K., Khoshnan, A., … Steffan, J. S. (2009). IKK phosphorylates Huntingtin and targets it for degradation by the proteasome and lysosome. *The Journal of Cell Biology*, *187*(7), 1083–1099. https://doi.org/10.1083/jcb.200909067

Turner, C., Cooper, J. M., & Schapira, A. H. V. (2007). Clinical correlates of mitochondrial function in Huntington's disease muscle. *Movement Disorders*, *22*(12), 1715–1721. https://doi.org/10.1002/mds.21540

Valekova, I., Jarkovska, K., Kotrcova, E., Bucci, J., Ellederova, Z., Juhas, S., … Kovarova, H. (2016). Revelation of the IFNα, IL-10, IL-8 and IL-1β as promising biomarkers reflecting immuno-pathological mechanisms in porcine Huntington's disease model. *Journal of Neuroimmunology*, *293*, 71–81. https://doi.org/10.1016/j.jneuroim.2016.02.012

van der Burg, J. M. M., Björkqvist, M., & Brundin, P. (2009). Beyond the brain: widespread pathology in Huntington's disease. *The Lancet. Neurology*, *8*(8), 765–774. https://doi.org/10.1016/S1474-4422(09)70178-4

van der Grond, J. (1995). Diagnosing testicular function using 31P magnetic resonance spectroscopy: a current review. *Human Reproduction Update*, *1*(3), 276–283. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/9187943

van der Grond, J., Laven, J. S., Lock, M. T., te Velde, E. R., & Mali, W. P. (1991). 31P magnetic resonance spectroscopy for diagnosing abnormal testicular function. *Fertility and Sterility*, *56*(6), 1136–1142. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/1743334

van der Grond, J., Laven, J. S., te Velde, E. R., & Mali, W. P. (1991). Abnormal testicular function: potential of P-31 MR spectroscopy in diagnosis. *Radiology*, *179*(2), 433–436.

https://doi.org/10.1148/radiology.179.2.2014287

van der Grond, J., Laven, J. S., van Echteld, C. J., Dijkstra, G., Grootegoed, J. A., de Rooij, D. G., & Mali, W. P. (1992). The progression of spermatogenesis in the developing rat testis followed by 31P MR spectroscopy. *Magnetic Resonance in Medicine*, *23*(2), 264–274. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/1549041

van der Grond, J., Van Pelt, A. M., van Echteld, C. J., Dijkstra, G., Grootegoed, J. A., de Rooij, D. G., & Mali, W. P. (1991). Characterization of the testicular cell types present in the rat by in vivo 31P magnetic resonance spectroscopy. *Biology of Reproduction*, *45*(1), 122–127. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/1652290

Van Raamsdonk, J. M., Murphy, Z., Selva, D. M., Hamidizadeh, R., Pearson, J., Petersén, Åsa, … Leavitt, B. R. (2007). Testicular degeneration in Huntington disease. *Neurobiology of Disease*, *26*(3), 512–520. https://doi.org/10.1016/j.nbd.2007.01.006

Van Raamsdonk, J. M., Pearson, J., Rogers, D. A., Bissada, N., Vogl, A. W., Hayden, M. R., & Leavitt, B. R. (2005). Loss of wild-type huntingtin influences motor dysfunction and survival in the YAC128 mouse model of Huntington disease. *Human Molecular Genetics*, *14*(10), 1379–1392. https://doi.org/10.1093/hmg/ddi147

Velier, J., Kim, M., Schwarz, C., Kim, T. W., Sapp, E., Chase, K., … DiFiglia, M. (1998). Wild-Type and Mutant Huntingtins Function in Vesicle Trafficking in the Secretory and Endocytic Pathways. *Experimental Neurology*, *152*(1), 34–40. https://doi.org/10.1006/exnr.1998.6832

Věžník Z., Švecová D., Zajícová A., Přinosilová P., 2004: Repetitorium spermatologie a andrologie a metodiky spermatoanalýzy. *VÚVeL, Brno, 197 s*

Vicari, E., Perdichizzi, A., De Palma, A., Burrello, N., D'Agata, R., & Calogero, A. E. (2002). Globozoospermia is associated with chromatin structure abnormalities: case report. *Human Reproduction (Oxford, England)*, *17*(8), 2128–2133. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/12151448

Vonsattel, J. P., & DiFiglia, M. (1998). Huntington disease. *Journal of Neuropathology and Experimental Neurology*, *57*(5), 369–384. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/9596408

Vonsattel, J. P., Myers, R. H., Stevens, T. J., Ferrante, R. J., Bird, E. D., & Richardson, E. P. (1985). Neuropathological classification of Huntington's disease. *Journal of Neuropathology and Experimental Neurology*, *44*(6), 559–577. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/2932539

Walker, F. O. (2007). Huntington's disease. *Lancet (London, England)*, *369*(9557), 218–228. https://doi.org/10.1016/S0140-6736(07)60111-1

Wellington, C. L., Ellerby, L. M., Gutekunst, C.-A., Rogers, D., Warby, S., Graham, R. K., … Hayden, M. R. (2002). Caspase cleavage of mutant huntingtin precedes neurodegeneration in Huntington's disease. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience*, *22*(18), 7862–7872. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/12223539

Wellington, C. L., Ellerby, L. M., Hackam, A. S., Margolis, R. L., Trifiro, M. A., Singaraja, R., … Hayden, M. R. (1998). Caspase cleavage of gene products associated with triplet expansion disorders generates truncated fragments containing the polyglutamine tract. *The Journal of Biological Chemistry*, *273*(15), 9158–9167. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/9535906

White, J. K., Auerbach, W., Duyao, M. P., Vonsattel, J.-P., Gusella, J. F., Joyner, A. L., & MacDonald, M. E. (1997). Huntingtin is required for neurogenesis and is not impaired by the Huntington's disease CAG expansion. *Nature Genetics*, *17*(4), 404–410. https://doi.org/10.1038/ng1297-404

Wild, E. J., & Tabrizi, S. J. (2017). Therapies targeting DNA and RNA in Huntington's disease. *The Lancet. Neurology*, *16*(10), 837–847. https://doi.org/10.1016/S1474-4422(17)30280-6

Yang, S., Chang, R., Yang, H., Zhao, T., Hong, Y., Kong, H. E., … Li, X.-J. (2017). CRISPR/Cas9-mediated gene editing ameliorates neurotoxicity in mouse model of Huntington's disease. *Journal of Clinical Investigation*, *127*(7), 2719–2724. https://doi.org/10.1172/JCI92087

ZELENÁ V., Fanfrdlová Z., Rektorová I.,(2009) Classic form of Huntington's disease manifested by asymmetric Parkinson's syndrome with rigidity responding to levodopa, *Neurol. pro praxi* 2009; 10(1): 50–53

Zuccato, C., Ciammola, A., Rigamonti, D., Leavitt, B. R., Goffredo, D., Conti, L., … Cattaneo, E. (2001). Loss of Huntingtin-Mediated BDNF Gene Transcription in Huntington's Disease. *Science*, *293*(5529), 493–498. https://doi.org/10.1126/science.1059581

Zuccato, C., Valenza, M., & Cattaneo, E. (2010). Molecular Mechanisms and Potential Therapeutical Targets in Huntington's Disease. *Physiological Reviews*, *90*(3), 905–981. https://doi.org/10.1152/physrev.00041.2009

Zvěřina, Jaroslav. *Sexuologie (nejen) pro lékaře*. Brno: CERM, 2003. 287 s. ISBN 80-7204-264-5.

8. PREHĽAD VŠETKÝCH RUKOPISOV V RÁMCI PHD. ŠTÚDIA

8.1. Publikácie v impaktovaných časopisoch

Monika Macakova, **Bozena Bohuslavova**, Petra Vochozkova, Antonin Pavlok, Miroslava Sedlackova, Daniela Vidinska, Klara Vochyanova, Irena Liskova, Ivona Valekova, Monika Baxa, Zdenka Ellederova, Jiri Klima, Stefan Juhas, Jana Juhasova, Jana Klouckova, Martin Haluzik, Jiri Klempir, Hana Hansikova, Jana Spacilova, Ryan Collins, Ian Blumenthal, Michael Talkowski, James F. Gusella, David S. Howland, Marian DiFiglia, Jan Motlik (2016)**Mutated Huntingtin Causes Testicular Pathology in Transgenic Minipig Boars**. *Neurodegenerative Diseases*, *16*(3–4), 245–259. https://doi.org/10.1159/000443665

IF (2016) 2,842

M. Jozefovicova, V. Herynek, F. Jiru, M. Dezortova, J. Juhásová, S. Juhas, J. Klíma, **B. Bohuslavova,** J. Motlik, M. Hájek (2015) **^{31}P MR Spectroscopy of the Testes and Immunohistochemical Analysis of Sperm of Transgenic Boars Carried N-terminal Part of Human Mutated Huntingtin,** *Česká a slovenská neurologie a neurochirurgie*, Cesk Slov Neurol N 2015; 78/111(Supplementum 2): 28-33,*DOI:* 10.14735/amcsnn20152S28

IF (2015) 0,209

Jana Krizova, Hana Stufkova, Marie Rodinova, Monika Macakova, **Bozena Bohuslavova**, Daniela Vidinska, Jiri Klima, Zdenka Ellederova, Antonin Pavlok, David S. Howland, Jiri Zeman, Jan Motlik and Hana Hansikova *(2017),* **Mitochondrial metabolism in large-animal model of huntington's disease: the hunt for biomarkers in the spermatozoa of presymptomatic minipigs** *Neuro-Degenerative Diseases*, *17*(4–5), 213–226. https://doi.org/10.1159/000475467

IF (2017) 2,842

Ardan T, Němcová L, **Bohuslavová B**, Klezlová A, Popelka Š, Studenovská H, Hrnčiarová E, Čejková J, Motlík J. (2015) **Reduced Levels of Tissue Inhibitors of Metalloproteinases in**

UVBIrradiated Corneal Epithelium *Photochem Photobiol*. 2016 Jun 18. doi: 10.1111/php.12612

IF (2015) 2,477

Daniela Vidinská, Petra Vochozková, Petra Šmatlíková, Taras Ardan, Štefan Juhás, Jana Juhásová, **Božena Bohuslavová,** Monika Baxa, Ivona Valeková Ján Motlík, Zdenka Ellederová **Gradual phenotype development in Huntington's disease transgenic minipig model at 24 months of age** Podaný v časopise *Neuro-Degenerative Diseases*

IF (2017) 2,842

Melvin M. Evers, Jana Miniarikova, Stefan Juhas, Astrid Valles-Sanchez, **Bozena Bohuslavova**, Jana Juhasova, Helena Kupcova Skalnikova, Petr Vodicka, Cynthia Brouwers, Bas Blits, Hana Kovarova, Zdenka Ellederova, Jan Motlik, Sander J. van Deventer, Harald Petry, Pavlina Konstantinova **AAV5-miHTT gene therapy demonstrates broad distribution and strong mutant huntingtin lowering in a Huntington´s disease minipig model** Podaný v časopise *Neuron Journal*
IF(2017) 14,024

8.2. Publikácie v knižných zborníkoch

Petra Rausova, Petra Vochozkova, Daniela Vidinska, Eva Hrnciarova, **Bozena Bohuslavova**, Monika Macakova, Ivona Valekova, Stefan Juhas, Taras Ardan, Petr Solc, Jan Motlik, Zdenka Ellederova (2017) **Porcine model of huntington's disease** Kapitola v knihe *Huntington disease*, (2017)

8.3. Publikácie v neimpaktovaných časopisoch

Gabriela Kocurová, Daniela Pallová, **Božena Bohuslavová**, Taras Ardan, Jan Motlík (2015)

Huntingtonova nemoc *Bioprospekt*

Božena Bohuslavová, Monika Mačáková (2015) **Testikulárna degenerácia pri huntingtonovej chorobe** *Bioprospekt*

8.4. Prezentácie prostredníctvom posterov

Monika Macakova, Antonin Pavlok, **Bozena Bohuslavova**, Hana Hansikova, Jana Spacilova, Miroslava Sedlackova, Monika Baxa,David S. Howland, Jan Motlik (2013) **Decrease of reproductive ability of HD transgenic boars continues** *2nd Conference on Large Animal Models of Huntington's disease Poster presentation*

Zdenka Ellederova, Monika Macakova, **Bozena Bohuslavova**, Jana Spacilova, Hana Hansikova, Stefan Juhas, Klara Vochyanova, Petra Vochozkova, Taras Ardan, Monika Baxa, Jan Motlik (2014) **Biomedical model of huntington´s disease: transgenic minipigs for n - terminal part of human mutated huntingtin** *Hereditary Disease Foundation - HD 2014, The Milton Wexler Celebration in Cambridge Poster presentation*

Vochozkova Petra , Macakova Monika , **Bohuslavova Bozena** , Sedlackova Miroslava , Juhas Stefan , Jan Motlik (2014) **Huntington disease phenotype in the testes of transgenic minipig boars for the human mutated huntingtin** *Morfology 2014, 7-9 september in Brno Poster presentation*

Jan Motlik, Monika Mačáková, **Božena Bohuslavová**, Antonín Pavlok, Hana Hansíková, Jana Spáčilová, Miroslava Sedláčkovád, Štefan Juhás, Jana Juhásová, Taras Ardan, Petra Vochozková, Monika Baxa, David Howland (2015) **Progress of huntington disease phenotype in transgenic minipigs for n-terminal part of human mutated huntingtin: invasive and non-invasive approaches** *10th Annual HD Therapeutics Conference in Palm Springs, United States, 23–26 Poster presentation*

Klima J, Vochozkova P, Juhasova J, **Bohuslavova B**, Macakova M, Motlik J, Juhas S (2015) **Spermatozoa immunophenotype markers asociated with porcine hd model** *The 3 rd Large animal models of neurodegenerative disease 8.11.-10.11.2015 in Liblice Poster presentation*

Bohuslavova B, Kucerova S, Macakova M, Ellederov Z, Motlik J (2015) **Behavioral and motoric testing of trangenic minipigs - focus on F0, F1 and F2 generations** *The 3 rd Large animal models of neurodegenerative disease 8.11.-10.11.2015 in Liblice Poster presentation*

Macakova M, **Bohuslavova B**, Vochozkova P, Baxa M, Ellederova Z, Sedlackova M, Liskova I, Valekova I, Vidinska D, Klima J, Juhas S, Motlik J (2015) **Testicular pathology in transgenic minipigs boars - in brief** *The 3 rd Large animal models of neurodegenerative disease 8.11.-10.11.2015 in Liblice Poster presentation*

Spacilova J, Rodinova M, Kratochvilova H, Ondruskova N, Macakova M, **Bohuslavova B,** Ellederova Z, Zeman J, Motlik J, Hansikova H (2015) **Mitochondrial functions in spermatozoa of minipig boars carrying transgene with the N-terminal part of human mutated huntingtin** *The 3 rd Large animal models of neurodegenerative disease 8.11.-10.11.2015 in Liblice Poster presentation*

Ellederova Z, Vidinska D, Macakova M, Kucerova S, **BohuslavovaB**, Sedlackova M, Liskova I, Valekova I, Baxa M, Ardan T, Juhas S, Motlik J (2015) **Phenotype development in tghd minipigs** – *The 3 rd Large animal models of neurodegenerative disease 8.11.-10.11.2015 in Liblice Abstrakt*

Bohuslavova B ,Kucerova S, Macakova M, Klempir J, Lavickova R,Krupicka R, Kauler J,Baxa M, Ellederova Z and Motlik J (2016) **Behavioral studies in libechov minipigs with huntington's disease changes in behavior, motor skills and learning** *The 9th EHDN Plenary Meeting 2016 15.9.2016-18.9.2016 in Haag Poster presentation*

Z. Ellederová, **B. Bohuslavová**, D. Vidinská, Š. Kučerova, M. Mačákova , P. Vochozkova, T.Ardan, R. Lavičková, J. Klempíř, M.Pokorný, S. Juhás , M. Rodinová, H. Hansíková, J. Motlik (2016**) Phenotype progression in tghd minipigs: behavioral and motoric impairments starting around six years of age, following after biochemical changes detected in younger animals** *CHDI foundation 22.2.-25.2.2016 http://dx.doi.org/10.1136/jnnp-2016-314597.91 Poster presentation*

Jana Miniarikova, Stefan Juhas, **Bozena Bohuslavova**, Jana Juhasova, Helena Kupcová Skalníková, Cynthia Brouwers, Astrid Vallés, Ralf Reilmann, Hana Kovarova, Zdenka Ellederova, Jan Motlík, Harald Petry, Pavlina Konstantinova (2017) **AAV5-mihtt gene therapy demonstrates broad vector distribution and strong mutant huntingtin lowering in huntington´s disease minipig model** *CHDI's 12th Annual HD Therapeutics Conference took place April 24 – 27, 2017, in Malta Poster presentation*

Bozena Bohuslavova, Monika Macakova , Monika Baxa , Stefan Juhas , Zdenka Ellederova Jan Motlik (2017) **Phenotypic study of Huntington's disease TgHD miinipigs: from reproductive changes to behavioral testing and gene therapy** *8 Th international Symphosium on experimental and clinical neurobiology 18-21 June 2017 in Košice Poster presentation*

Monika Baxa, Bozena Bohuslavova, Sarka Kucerova, Monika Macakova, Jiri Klempir, Jan Motlik, Zdenka Ellederova (2017) **Battery of tests assessed to monitor motor and cognitive functions of transgenic Huntington´s disease minipig model** *4th Large Animal Models of Neurodegenerative Diseases 22-24 October, Liblice Poster presentation*

J. Klíma, J. Juhásová, Š. Juhás, **B. Bohuslavová**, J. Motlík and Z. Ellederová (2017) **Putative CD68 as a neurodegeneration marker in porcine TgHD cortex** *4th Large Animal Models of Neurodegenerative Diseases 22-24 October, Liblice Poster presentation*

Krizova J, Stufkova H, Rodinova M, Macakova M, **Bohuslavova B**, Vidinska D,Klima J, Ellederova Z, Zeman J, Motlik J, Hansikova H (2017) **Biomarkers in the spermatozoa of presymptomatic minipig model for Huntington's disease** *4th Large Animal Models of Neurodegenerative Diseases 22-24 October, Liblice Poster presentation*

Rodinova M, Krizova J, Dosoudilova Z, Stufkova H, Ondruskova N, **Bohuslavova B**, Juhas S,
Ellederova Z, Zeman J, Motlik J, Hansikova H (2017) **The energy metabolism dysfunction in skeletal muscle of Huntington's disease minipig model** *4th Large Animal Models of Neurodegenerative Diseases 22-24 October, Liblice Poster presentation*

Valekova Ivona, Butalova Nikola, Kovarova Hana, **Bohuslavova Bozena**, Motlik Jan and Ellederova Zdenka (2017) **Immuno-inflammatory studies in minipig model bearing the N-terminal human mutant huntingtin** *4th Large Animal Models of Neurodegenerative Diseases 22-24 October, Liblice Poster presentation*

9. PREHĽAD PRÍLOH

Monika Macakova, **Bozena Bohuslavova**, Petra Vochozkova, Antonin Pavlok, Miroslava Sedlackova, Daniela Vidinska, Klara Vochyanova, Irena Liskova, Ivona Valekova, Monika Baxa, Zdenka Ellederova, Jiri Klima, Stefan Juhas, Jana Juhasova, Jana Klouckova, Martin Haluzik, Jiri Klempir, Hana Hansikova, Jana Spacilova, Ryan Collins, Ian Blumenthal, Michael Talkowski, James F. Gusella, David S. Howland, Marian DiFiglia, Jan Motlik (2016)**Mutated Huntingtin Causes Testicular Pathology in Transgenic Minipig Boars**. *Neurodegenerative Diseases*, *16*(3–4), 245–259. https://doi.org/10.1159/000443665

IF (2016) 2,842

M. Jozefovicova, V. Herynek, F. Jiru, M. Dezortova, J. Juhásová, S. Juhas, J. Klíma, **B. Bohuslavova,** J. Motlik, M. Hájek (2015) **^{31}P MR Spectroscopy of the Testes and Immunohistochemical Analysis of Sperm of Transgenic Boars Carried N-terminal Part of Human Mutated Huntingtin,** *Česká a slovenská neurologie a neurochirurgie*, Cesk Slov Neurol N 2015; 78/111(Supplementum 2): 28-33,*DOI:* 10.14735/amcsnn20152S28

IF (2015) 0,209

Jana Krizova, Hana Stufkova, Marie Rodinova, Monika Macakova, **Bozena Bohuslavova**, Daniela Vidinska, Jiri Klima, Zdenka Ellederova, Antonin Pavlok, David S. Howland, Jiri Zeman, Jan Motlik and Hana Hansikova *(2017),* **Mitochondrial metabolism in large-animal model of huntington's disease: the hunt for biomarkers in the spermatozoa of presymptomatic minipigs** *Neuro-Degenerative Diseases*, *17*(4–5), 213–226. https://doi.org/10.1159/000475467

IF (2017) 2,842

10. CURRICULUM VITAE

MVDr. Božena Bohuslavová

e-mail:

bohuslavova@iapg.cas.cz

Pozície:

9/2013 –súčasnosť: Centrum Pigmod, Liběchov , ČR

Laboratoř bunkové regenerace a plasticity

PhD študent

9/2013 – súčasnosť: Ústav živočíšne fyziologie a genetiky AV ČR Liběchov

Laboratoř bunkové regenerace a plasticity

PhD študent

9/2011-6/2012 : Diplomant na katedre biológie a genetiky Univerzity veterinárske lekárstva a farmácie v Košiciach, SR

Vzdelanie:

10/2013 súčasnosť: Karlova univerzita v Prahe, Přírodovědecká fakulta

Študijný progra:: Bunková a vývojová biologia

PhD štúdium

2006-2013 : Univerzita veterinárskeho lekárstva a farmácie v Košiciach, SR

Študijný program: Všeobecné veterinárske lekárstvo

MVDr. štúdium

2002-2006 : Gymnázium Konštantínova 2, Prešov

Doplnkové vzdelanie.

06/2014	:	Kurz základov vedeckej práce
02/2015	:	Kurz práce s laboratornymi zvieratami, Osvědčení o odborné zpusobilosti k navrhovaní pokusú a projektú pokusu, evidečné číslo CZ 02939
06/2016	:	Kurz spracovanie a analýza mikroskopických obrázkov v biomedicíne
31.3.2017	:	Osvědčení o způsobilosti pro řidiče a pruvodce, evidečné číslo CZ 07234

Vedecké členstvá:

EHDN – Europen Huntington´s Disease Network

Jazykové zručnosti:

Slovenský jazyk:	rodný
Český jazyk:	pasívne
Anglický jazyk:	stredne pokročilý
Franzúzsky jazyk:	základy

Original Paper

Neuro–degenerative Diseases

Neurodegener Dis 2016;16:245–259
DOI: 10.1159/000443665

Received: June 1, 2015
Accepted after revision: December 23, 2015
Published online: March 10, 2016

Mutated Huntingtin Causes Testicular Pathology in Transgenic Minipig Boars

Monika Macakova[a, c] Bozena Bohuslavova[a, c] Petra Vochozkova[a, c] Antonin Pavlok[a] Miroslava Sedlackova[b] Daniela Vidinska[a, c] Klara Vochyanova[a, c] Irena Liskova[a, d] Ivona Valekova[a, c] Monika Baxa[a, c] Zdenka Ellederova[a] Jiri Klima[a] Stefan Juhas[a] Jana Juhasova[a] Jana Klouckova[e] Martin Haluzik[e] Jiri Klempir[d] Hana Hansikova[f] Jana Spacilova[f] Ryan Collins[g] Ian Blumenthal[g] Michael Talkowski[g] James F. Gusella[g] David S. Howland[i] Marian DiFiglia[h] Jan Motlik[a]

[a]Laboratory of Cell Regeneration and Plasticity, Institute of Animal Physiology and Genetics, Czech Academy of Science, Libechov, [b]Department of Histology and Embryology, Faculty of Medicine, Masaryk University in Brno, Brno, [c]Department of Cell Biology, Faculty of Science, and [d]Department of Neurology and Centre of Clinical Neuroscience, First Faculty of Medicine, Charles University in Prague, and [e]3rd Department of Medicine, Department of Endocrinology and Metabolism, and [f]Laboratory for Study of Mitochondrial Disorders, Department of Pediatrics and Adolescent Medicine, First Faculty of Medicine, Charles University and General University Hospital in Prague, Prague, Czech Republic; [g]Center for Human Genetic Research, and [h]Department of Neurology, Massachusetts General Hospital, Boston, Mass., and [i]CHDI Foundation, Princeton, N.Y., USA

Key Words
Huntington's disease · Pig model · Mutant huntingtin · Spermatozoa · Testes · Degeneration

Abstract
Background: Huntington's disease is induced by CAG expansion in a single gene coding the huntingtin protein. The mutated huntingtin (mtHtt) primarily causes degeneration of neurons in the brain, but it also affects peripheral tissues, including testes. ***Objective:*** We studied sperm and testes of transgenic boars expressing the N-terminal region of human mtHtt. ***Methods:*** In this study, measures of reproductive parameters and electron microscopy (EM) images of spermatozoa and testes of transgenic (TgHD) and wild-type (WT) boars of F1 (24–48 months old) and F2 (12–36 months old) generations were compared. In addition, immunofluorescence, immunohistochemistry, Western blot, hormonal analysis and whole-genome sequencing were done in order to elucidate the effects of mtHtt. ***Results:*** Evidence for fertility failure of both TgHD generations was observed at the age of 13 months. Reproductive parameters declined and progressively worsened with age. EM revealed numerous pathological features in sperm tails and in testicular epithelium from 24- and 36-month-old TgHD boars. Moreover, immunohistochemistry confirmed significantly lower proliferation activity of spermatogonia in transgenic testes. mtHtt was highly expressed in spermatozoa and testes of TgHD boars and localized in all cells of seminiferous tubules. Levels of fertility-related hormones did not differ in TgHD and WT siblings. Genome analysis confirmed that insertion of the lentiviral construct did not interrupt any coding sequence in the pig genome. ***Conclusions:*** The sperm and testicular degeneration of TgHD boars is caused by gain-of-function of the highly expressed mtHtt.

Monika Macakova and Bozena Bohuslavova contributed equally to this work.

KARGER

E-Mail karger@karger.com
www.karger.com/ndd

1660–2854/16/0164–0245$39.50/0

Jan Motlik
Laboratory of Cell Regeneration and Plasticity
Institute of Animal Physiology and Genetics
Rumburska 89, CZ–27721 Libechov (Czech Republic)
E-Mail motlik@iapg.cas.cz

Introduction

Huntington's disease (HD) is a neurodegenerative disorder caused by the expansion of CAG repeat in the gene encoding the huntingtin protein (Htt), which is expressed in most tissues. The onset of the disease is usually in the mid-thirties. The main target is the central nervous system, but it has an impact on the whole body. There is no available curative treatment to date. Even the pathogenesis of the disease is not well understood. Nevertheless, it is well known that mutated Htt (mtHtt) forms cytoplasmic and nuclear aggregates, particularly in the cerebral cortex, striatum and lateral hypothalamus [1]. Many rodent models of HD that express either truncated or full-length human mutant Htt display differences in the onset and severity of phenotypes. Rodent models have collectively provided valuable information related to target validation and drug therapy [2–4]. However, large animal models are expected to simulate the disease more faithfully and moreover enable the usage of medical techniques and equipment applicable for human patients [5].

Minipigs represent a desirable model for longitudinal safety studies and preclinical drug trials to fill the gap between rodent models and patients [6, 7]. The advantage of minipigs is their resemblance with the human brain as well as with the whole body in terms of size, anatomy and physiology. There is a 96% homology between porcine and human huntingtin genes and proteins [8] that provides further impetus to use the minipig as a model of HD. Therefore, a transgenic minipig model was generated using microinjection of a lentiviral vector encoding the N-terminal (1–548 aa) of human Htt containing 145 CAG/CAA repeats under the control of the human HTT promoter [9]. The mtHtt gene with 124 glutamines was incorporated into chromosome 1 (1q24–q25), and the expression of mtHtt was detected in numerous peripheral tissues. Successful germ line transmission occurred through 4 successive generations inheriting the mutation in Mendelian ratio [9].

Even though the neurological phenotype of HD patients is the most prominent, the first sign of phenotype development in TgHD boars of F1 generation was reproductive failure, starting at the age of 13 months [9]. Interestingly, among all organs, the testes display the most comparable gene expression pattern to the brain [10]. In accordance with this finding, the expression of mtHtt in R6/2 and YAC128 mouse models of HD results in atrophy of the brain and testes [11–13]. Closer examination of the testes in YAC128 mice revealed disorganized seminiferous epithelium and a reduced number of germ cells. YAC72 mice expressing mtHtt but lacking endogenous Htt ($YAC72^{-/-}$) revealed an even more severe phenotype resulting in infertility with aspermia and massive apoptotic cell death in the testes [14]. Also, a detailed testes examination in HD patients documented testicular abnormalities as well as reduced numbers of germ cells and abnormal morphology of seminiferous tubules [13].

The question arises whether the defect in testes is caused by the presence of mtHtt in testes or by a defect in neurons responsible for hormonal changes. In R6/2 mice, a secondary effect due to the decreased level of gonadotropin-releasing hormone (GnRH)-immunoreactive neurons was suggested. Only 10% of GnRH neurons remained in R6/2 mice by 9 weeks of age, while testicular atrophy and infertility were detected at 12 weeks of age together with a decrease of testosterone levels in serum and testes [11]. Nonetheless, the direct effect of mtHtt was not considered. On top of that, a previous paper showed testicular atrophy in R6/2 mice by 4 weeks of age [15], a week prior to the start of GnRH neuronal loss. In the YAC128 mouse model, testicular degeneration developed between 9 and 12 months of age, but even at 12 months, there is no evidence for decreased testosterone levels in urinary and plasma samples or loss of GnRH neurons in the hypothalamus [13].

In this paper, we followed reproductive parameters of TgHD and WT minipig boars from F1 and F2 generations in order to describe their sperm and testicular pathology phenotype. Furthermore, we investigated whether the phenotype is caused by the primary effect of mtHtt. We ruled out hormonal changes or interruption of any coding sequence during insertion of the lentiviral construct. Here we show evidence for morphological and functional defects in sperm and testes over two generations of TgHD minipigs that accrue before the neurology defects and hormonal changes, suggesting a direct toxic consequence of the expressed N-terminal mtHtt.

Materials and Methods

Animals

Transgenic minipigs with the N-terminal part of human mtHtt [9] were studied. Transgenic boars (n = 17) and their wild-type male controls (n = 13) were used in experiments. All components of this study were carried out in accordance with the Animal Care and Use Committee of the Institute of Animal Physiology and Genetics and were conducted according to current Czech regulations and guidelines for animal welfare and with approval by the State Veterinary Administration of the Czech Republic.

For an overview of the animals used in experiments see supplementary material SM 1 (for all online suppl. material, see www.karger.com/doi/10.1159/000443665).

Spermatozoa Collection, Measurement of Sperm Parameters and in vitro Fertilization Test

Semen was collected from boars of F1 (age 24–48 months, n = 4) and F2 (age 12–36 months, n = 8) generations. All samples were evaluated using a sperm cell analyzer (Microptic, Spain) immediately after collection. The number of spermatozoa per ejaculate and the motility and progressivity of the spermatozoa were assessed. In vitro fertilization tests were done as previously described [9].

Preparation of Testicular Tissue

Testicular tissue was obtained from boars of F2 generation aged 24 (n = 2) and 36 months (n = 4). Animals were perfused under deep anesthesia with cold PBS. The tissue of the right testis was fixed in 4% paraformaldehyde followed by cryoprotection in 30% sucrose in 0.1 M PBS and used for immunohistochemistry and electron microscopy (EM). The tissue of the left testis was used for SDS-PAGE and Western blot.

Electron Microscopy

Small blocks of testicular tissue and ejaculate samples were fixed in 300 mM glutaraldehyde (Sigma-Aldrich) in 100 mM cacodylate buffer for 2 h at room temperature (RT), washed in the same buffer and postfixed in 40 mM osmium tetroxide (Polysciences) in 100 mM cacodylate buffer for 1 h at RT. Samples of testicular tissue were embedded in araldite resin (Durcupan ACM; Sigma-Aldrich) after rinsing in cacodylate buffer and dehydration in ethanol. Ejaculate samples were embedded in agar blocks, dehydrated in ethanol and embedded in araldite resin (Durcupan ACM; Sigma-Aldrich).

For immunohistochemical analyses, samples of ejaculate were washed in PBS and fixed in 4% paraformaldehyde with 0.1% glutaraldehyde in PBS. The samples were embedded in agar blocks, dehydrated in ethanol and embedded in LR white resin (Sigma-Aldrich). Samples were incubated with mouse anti-polyglutamine monoclonal primary antibody (MAB1574; Millipore; 1:50) overnight at 4°C. Then the sections were rinsed in PBS and incubated with anti-mouse IgG-Gold antibody (10 nm gold particles; G7652; Sigma-Aldrich; 1:40) for 2 h at RT.

In all of the EM analyses, 60-nm-thick sections were cut using a Leica EM UC6 ultramicrotome and stained with uranyl acetate and lead citrate. Sections were examined under an FEI Morgagni 268D electron microscope (FEI Company, The Netherlands) at 70 kV.

Immunofluorescence and Immunohistochemistry

Spermatozoa were spotted onto clean slides using cytospin (800 *g*, 5 min). Spermatozoa were fixed and permeabilized with ice-cold absolute methanol for 5 min and then with acetone for 30 s. Slides were blocked with 5% goat serum and 5% milk for 30 min at RT. Sections were incubated with mouse anti-polyQ monoclonal antibody (3B5H10; Sigma Aldrich; 1:500) for 2 h at 4°C, and then Alexa Fluor 488-conjugated goat anti-mouse antibody (A21424; Invitrogen; 1:500) was applied for 1 h at RT. DAPI was added to the mounting medium.

Frozen testicular tissue was cut using a Leica CM1950 cryostat. Testicular sections (5 μm thick) were mounted on slides coated with 2% (3-aminopropyl) triethoxysilane in acetone (Sigma-Aldrich). Slides were heated for 10 min at 0.7 bar overpressure in 0.01 M sodium citrate buffer (pH 6.0) using a pressure cooker (Steba, Germany) for antigen retrieval. Sections were blocked with 10% goat or donkey serum and stained with mouse anti-PCNA monoclonal antibody (ab29; Abcam; 1:2,000) and rabbit anti-Ki67 monoclonal antibody (ab16667; Abcam; 1:1,000) or rabbit anti-Htt monoclonal antibody (EPR5526; Abcam; 1:250) overnight at 4°C. Sections stained with EPR5526 were further treated with Alexa Fluor 647-conjugated goat anti-rabbit antibody (Amersham; 1:500) for 1 h at RT and followed by mounting medium containing DAPI. Other sections were incubated with sheep anti-mouse biotinylated antibody or donkey anti-rabbit biotinylated antibody (Amersham; 1:200) for 1 h at RT followed by incubation with an avidin-peroxidase complex (Vector ABC Elite) and 3,3′-diaminobenzidine tetrahydrochloride (DAB; Sigma) chromogen. Sections were dehydrated in graded ethanol, cleared in xylene and then coverslipped using DPX. Slides were digitalized using a scanning microscope (Olympus BX) and images were edited using VS-120 software. Statistical analyses were performed using GraphPad Prism 5.0 software (one-way ANOVA with Duncan's post hoc test). PCNA-positive and Ki67-positive cells were counted in 20–30 seminiferous tubules per animal.

SDS-PAGE and Western Blot

Testes were homogenized in liquid nitrogen using a mortar. Spermatozoa and homogenized testes were lysed in RIPA buffer (150 mM NaCl, 1% NP-40, 0.5% deoxycholate, 0.1% SDS, 50 mM Tris-HCl pH 8, inhibitors of phosphatases and proteases), sonicated, and centrifuged at 10,000 *g* for 10 min at 4°C. Samples (20 μg of total protein) were loaded onto 3–8% Tris-acetate gel (EA03755; LifeTech) and run at 150 V. Gel was transferred onto nitrocellulose membrane (IB301001; LifeTech) at 250 mA. Membranes were blocked in 5% skimmed milk, and probed overnight with anti-Htt antibody (EPR5526; Abcam; 1:30,000 or AB1; Sigma Aldrich; 1:000), or anti-polyQ antibody (3B5H10; Sigma Aldrich; 1:3,000), tubulin staining was used as loading control (anti-tubulin; Sigma Aldrich; 1:10,000). Secondary antibody conjugated with HRP (anti-mouse, 711-035-152; Jackson ImmunoResearch; 1:10,000 or anti-rabbit, 711-035-152; Jackson ImmunoResearch; 1:10,000) was used. Light reaction was induced by ECL (RPN2232; GE Healthcare) and the signal was captured on CL-Xposure films (34091; Thermo Scientific).

Hormonal Assay

Blood samples were collected 5 times from age-matched TgHD (n = 15) and WT (n = 8) boars (aged 7–30 months). The samples were allowed to clot for 60 min at RT, and centrifuged twice (1,500 *g*, 10 min, 4°C). Serum levels of testosterone, luteinizing hormone (LH) and inhibin-α were determined by commercial ELISA kits (CSB-E06796p, CSB-E06791p, CSB-E12870p, CSB-EL-011718PI; CUSABIO, Wuhan, China). All measurements were performed in duplicate and according to the manufacturers' protocols. Statistical analysis was done using the Kolmogorov-Smirnov normality test followed by the unpaired t test.

Jumping Library Whole-Genome Sequencing

Customized sequencing libraries were constructed based on published protocols [16] and sequenced with paired-end 50-bp reads on an Illumina HiSeq2500. Library barcodes were demulti-

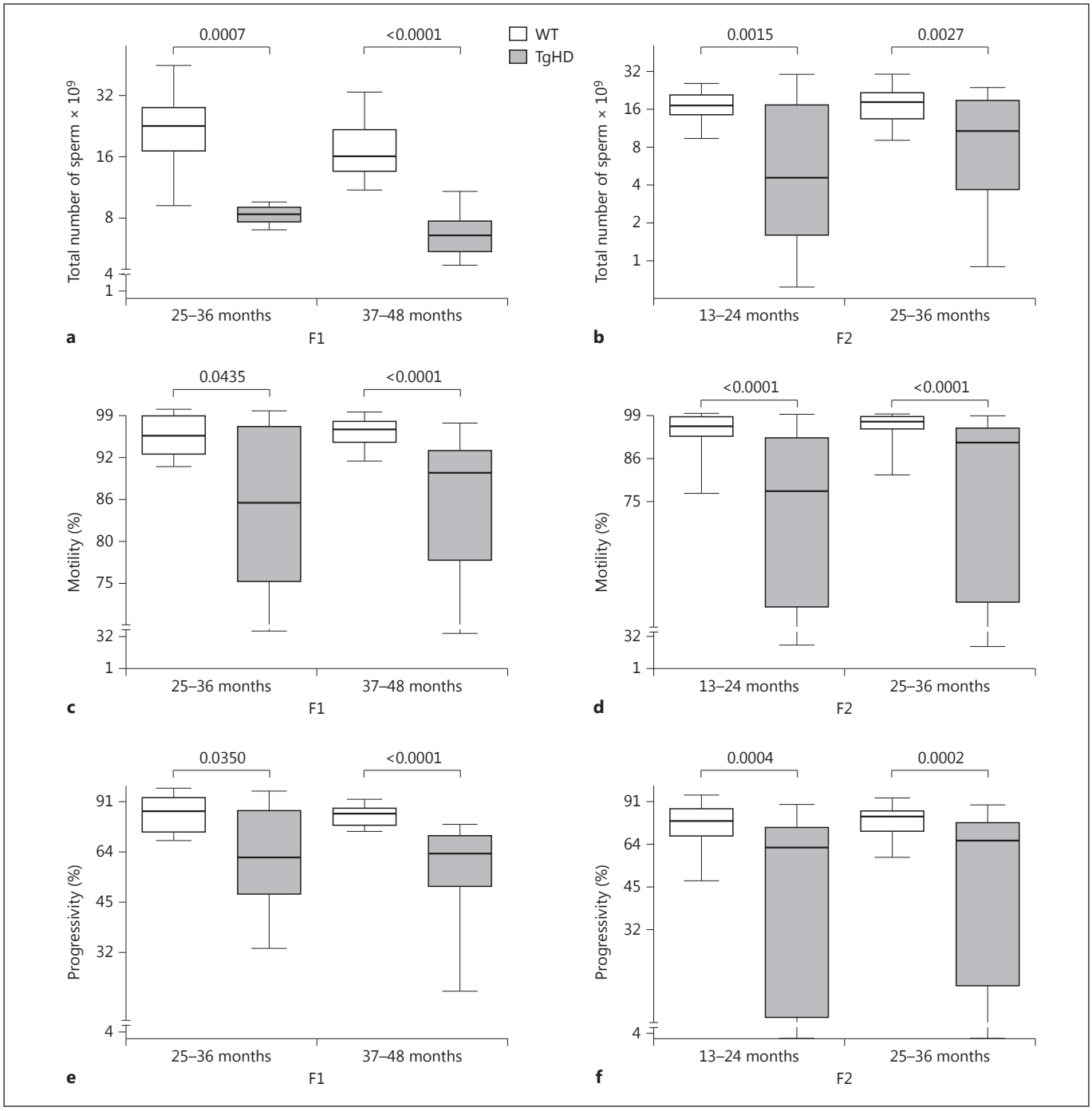


Fig. 1. Sperm parameters. **a** Sperm count in the semen samples of TgHD boars from F1 generation collected at the age of 25–36 and 37–48 months was significantly decreased in comparison with control animals. **b** Number of sperms in ejaculate of F2 TgHD boars collected at the age of 13–24 and 25–36 months was also lower in comparison with their WT littermates. Motility of the sperms was decreased in both F1 (**c**) and F2 (**d**) generations in all tested ages. Also, progressivity of the sperms was lower in TgHD animals from F1 (**e**) and F2 (**f**) generations. Data were analyzed using the Kolmogorov-Smirnov normality test followed by the Mann-Whitney U test. $p < 0.05$ was considered significant.

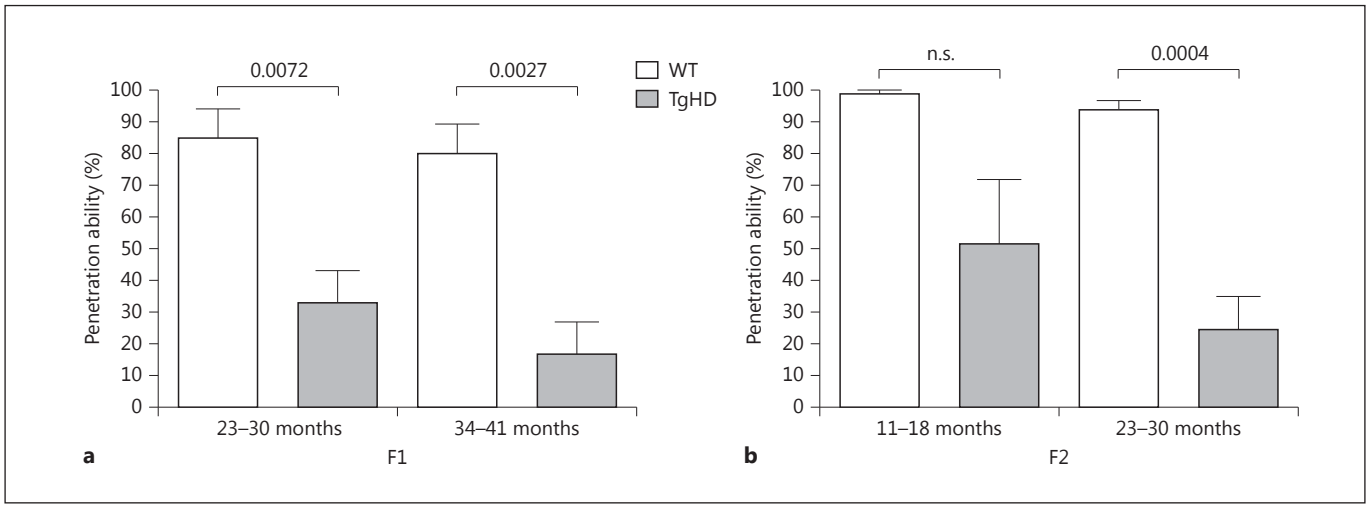


Fig. 2. Sperm penetration ability. **a** The ability to penetrate into oocytes is decreased in sperms of F1 boars at the age of 23–30 months, and also at the age of 34–41 months. The most striking difference in the penetration ability between WT and TgHD F2 boars was at the age of 23–30 months. **b** Such alterations were observed as well in animals from F2 generation who reached the age of 11–18 months ($p = 0.0608$). Each penetration test was repeated at least 5 times. Data were analyzed using the Kolmogorov-Smirnov normality test followed by the Mann-Whitney U test. $p < 0.05$ was considered significant.

plexed with CASAVA v1.7. Read quality was assessed with FastQC v0.11.2 (http://www.bioinformatics.babraham.ac.uk). Quality and adapter trimming was performed with TrimGalore v0.3.7 (http://www.bioinformatics.babraham.ac.uk). Reads were aligned to a modified version of *Sus scrofa* reference genome assembly Sscrofa10.2.74 (GCA_000003025.4; http://www.ensembl.org/Sus_scrofa) that included the full pHIV1-HD548aa-145Q vector sequence. Reads were aligned with BWA-backtrack v0.7.10-r789 [17]. Duplicates were marked with Picard Tools MarkDuplicates v0.1.111 (http://picard.sourceforge.net). All alignment manipulations, including sorting and indexing, was performed with sambamba v0.4.6 [18]. Alignment quality was assessed using Picard Tools, Samtools v1.0 and BamTools v2.2.2 [19, 20]. All chimeric read pairs mapping from endogenous reference sequences to the transgene or vector backbone sequences were isolated and clustered using our published algorithms *BamStat* and *ReadPairCluster* [21–23]. An independent algorithm, DELLY, was used to corroborate integration sites detected by principal methods [24]. Actual sequences of the integration junctions were determined by PCR and Sanger sequencing.

Results

Sperm Pathology of TgHD Boars

We showed altered reproduction parameters in 2 TgHD boars of F1 generation starting at the age of 13 months [9] as a potential HD phenotype in our porcine model. However, detailed analysis of a larger cohort of animals was needed to investigate the basis for the decline in fertility. We provided evidence on sperm reproductive parameters of TgHD and WT boars from F1 (24–36 months old) and F2 (12–36 months old) generations. Animals in the compared groups did not yet vary in weight, size or their motor movements.

Semen of TgHD and WT animals was collected and characterized using a sperm cell analyzer. Sperm count, motility and progressivity were evaluated. All parameters measured in semen samples of TgHD boars significantly decreased at around 13 months in both generations (fig. 1) and persisted at a low level with increasing age. In vitro fertilization tests showed a continuous decreased ability of TgHD sperms to penetrate the oocytes (fig. 2).

EM analysis of semen samples revealed altered morphology of spermatozoa between TgHD and WT boars. Structural anomalies of spermatozoa were much more numerous in TgHD samples. These abnormalities were more pronounced in the F2 generation. Nearly all the spermatozoa of TgHD animals of F2 generation had a cytoplasmic droplet (most often proximal; fig. 3a). Severe structural alterations in TgHD spermatozoa were localized mainly in the connecting piece and midpiece of the tail. Abnormalities were manifested as deformation of the mitochondrial sheath in the tail midpiece and also other tail structures. Common findings were folded or coiled tails, and sometimes a double or triple axoneme with fused mitochondrial sheaths (fig. 3b, d). Deformity of the

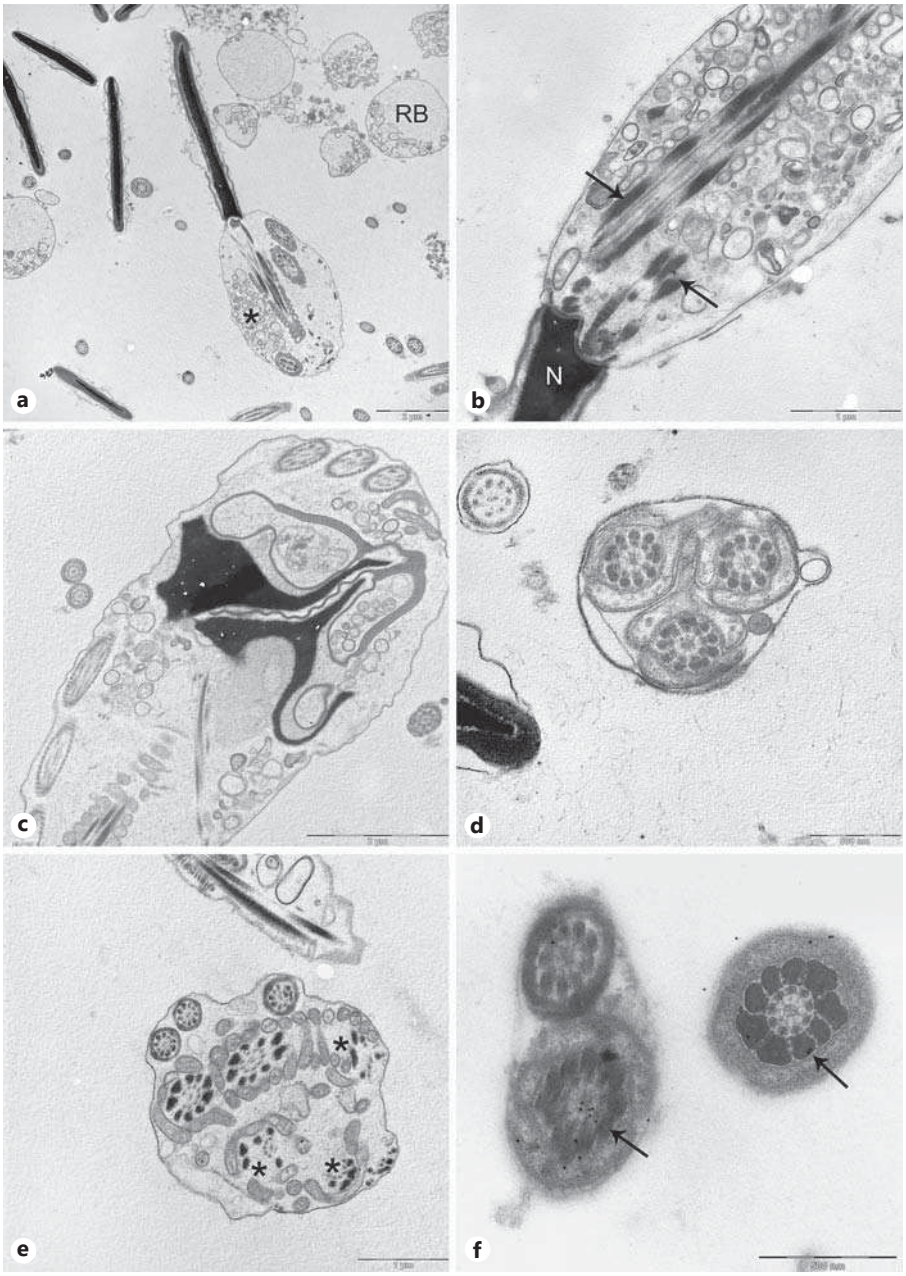


Fig. 3. Fresh ejaculate of TgHD boar. RB = Residual bodies; N = neck. **a** Spermatozoon with proximal cytoplasmic droplet (asterisk) and residual bodies. **b** Spermatozoon with cytoplasmic droplet and double neck and tail structures (arrows). **c** Fully deformed spermatozoon. **d** A tail of a spermatozoon with triple axoneme and fused mitochondrial sheaths. **e** Connecting piece of tail of a spermatozoon with multiple and totally disorganized axoneme and mitochondrial sheaths (asterisk). **f** Immunocytochemical reaction. 10-nm gold particles (arrows) indicate the presence of polyglutamine-containing proteins.

nucleus associated with incomplete chromatin condensation and abnormal acrosome occurred occasionally (fig. 3c). Instability of acrosomes was in some cases manifested by a precocious acrosomal reaction. Proximal cytoplasmic droplets were often associated with disorganized mitochondrial sheaths (fig. 3e). In the F2 generation, there was a total absence of residual bodies in the ejaculate.

Testicular Pathology of TgHD Boars

After having demonstrated the sperm pathology of TgHD boars, we sacrificed one pair of 24-month-old and 2 pairs of 36-month-old animals from the F2 generation to perform the morphological analysis of testes.

At the age of 24 months, degenerative changes in seminiferous epithelium were more frequent in the TgHD boar in comparison with the wild-type one. Apoptosis was seen in supporting Sertoli cells as well as cells of sper-

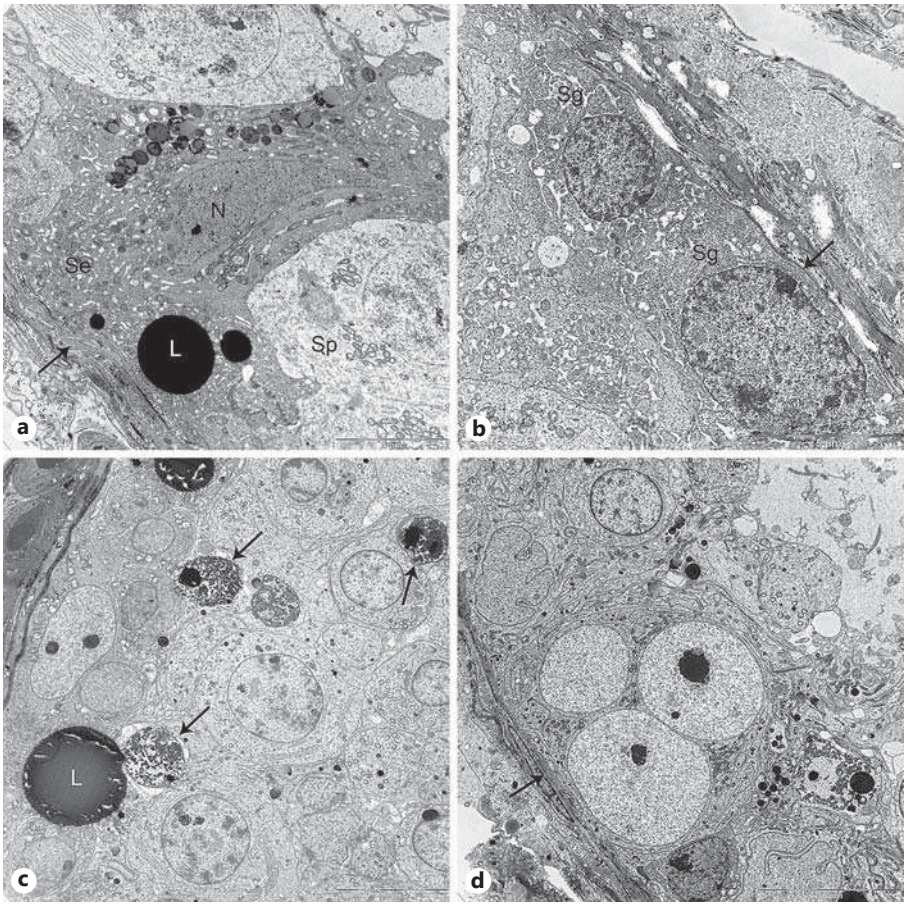


Fig. 4. Seminiferous epithelium of TgHD boar at the age of 24 months. Se = Sertoli cell undergoing apoptosis; N = nuclear structure; Sp = spermatocyte; L = lipid droplet; Sg = spermatogonia. **a** Sertoli cell undergoing apoptosis – increasing cytoplasm density, vacuolation, altered nuclear structure. Note spermatocyte and large lipid droplet. **b** Spermatogonia undergoing apoptosis. Note basal lamina (arrow). **c** Degenerated spermatocytes (arrows). Note large lipid droplet. **d** Spermatogonia with 3 nuclei. Note basal lamina (arrow).

matogenic lineage. Degeneration of Sertoli cells was characterized by increased density and vacuolation of cytoplasm, dilatation of endoplasmic reticulum, structural alterations of the nuclei and swollen mitochondria (fig. 4a). Degeneration of spermatogonia was manifested by cell shrinkage, increased chromatin condensation in the nucleus, dilatation of endoplasmic reticulum and swollen mitochondria with defects of their internal structure (fig. 4b). In addition, other cells of the spermatogenic lineage were gradually degenerated (fig. 4c). Strongly reduced epithelium of the seminiferous tubules, restricted to the Sertoli cells, spermatogonia and the sparsely occurring spermatocytes and spermatids were occasionally observed. Multinucleated spermatogenic cells including spermatogonia (fig. 4d) were frequently recognized. The thick basal lamina was made up of several layers (2–3 laminae densae).

At the age of 36 months, the rate of degenerative changes in the testicular samples of 2 transgenic individuals was different. The changes were less pronounced in boar K104 than in boar K63 and resembled those of a TgHD boar at the age 24 months. Morphology of seminiferous tubules in boar K63 showed a significant reduction of spermatogenesis. In some of the tubules, spermatogenesis was preserved (but only to a limited extent; (fig. 5a); other tubules contained only Sertoli cells (fig. 5b). In the tubules with preserved spermatogenesis, the degenerative changes were detected in both Sertoli cells (fig. 5d) and spermatogenic elements, including the spermatogonia. Degenerative changes exhibited characteristics typical for early or advanced apoptosis – the increased density of cytoplasm associated with its vacuolization, swollen mitochondria, dilated endoplasmic reticulum and clumps of heterochromatin in the nucleus. The basal lamina was thick and made up of several layers (2–3 laminae densae). Tubules without spermatogenic cells were lined with Sertoli cells exclusively, which, unlike the tubules with preserved spermatogenesis, hold signs of degeneration only in exceptional cases. Sertoli cells contained extreme amounts of endoplasmic reticulum, which

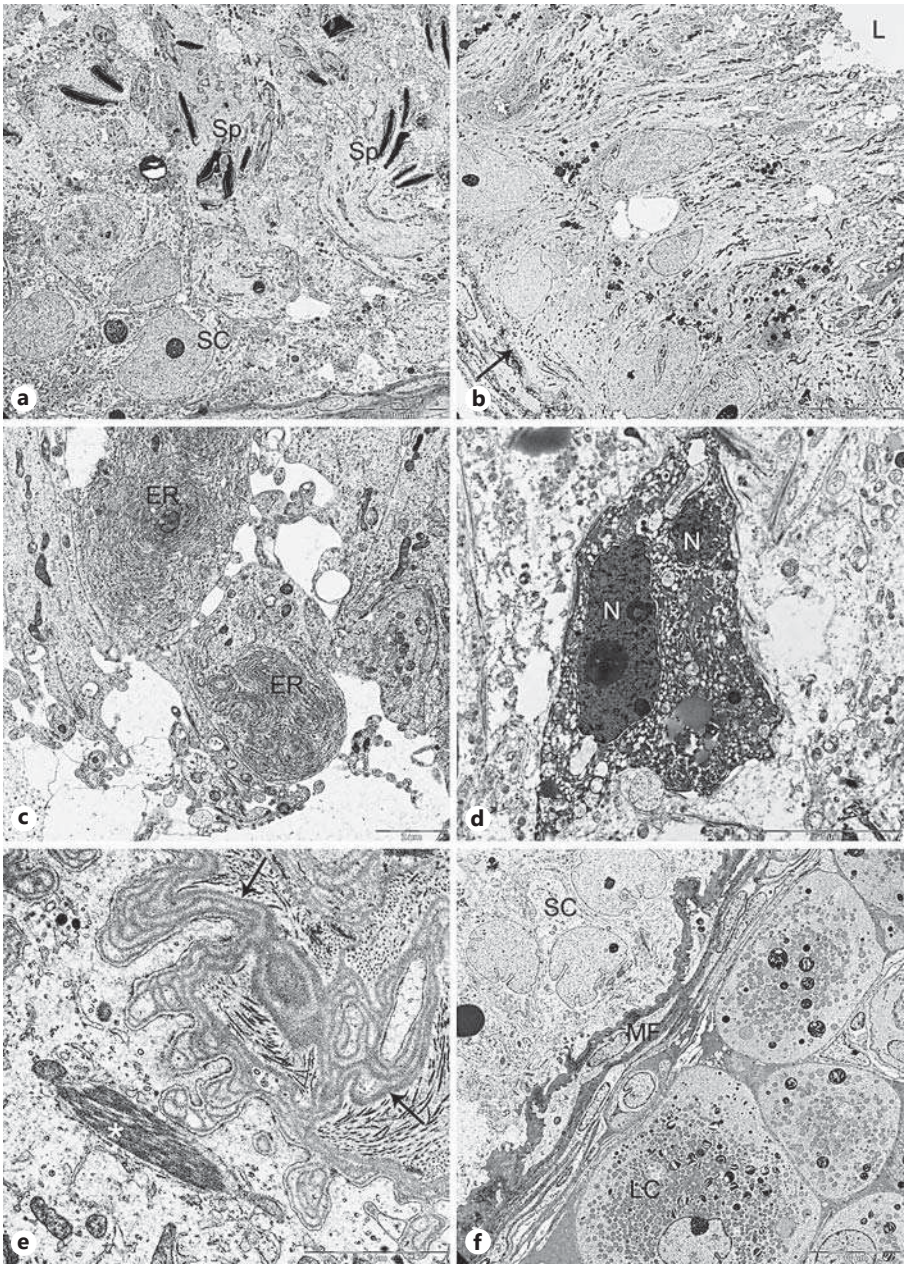


Fig. 5. Seminiferous epithelium of TgHD boar at the age of 36 months. SC = Sertoli cell; Sp = spermatids; L = lumen of tubule; ER = endoplasmic reticulum; N = nucleus of Sertoli cell; MF = myofibroblasts; LC = Leydig cells. **a** Preserved but reduced spermatogenesis. Note Sertoli cells and spermatids. **b** Seminiferous epithelium containing only intact Sertoli cells, with no spermatogenic elements. Note basal lamina (arrow) and lumen of tubule. **c** Sertoli cells of the same tubule – concentric cisternae of endoplasmic reticulum in apical cytoplasm. **d** Sertoli cell undergoing apoptosis in a tubule with preserved spermatogenesis. Note nucleus of Sertoli cell. **e** Basal part of a Sertoli cell with Charcot-Böttcher crystal (asterisk). Note split and undulated basal lamina (arrows). **f** The lamina propria of a tubule with absent spermatogenesis. Note myofibroblasts. Numerous Leydig cells occupy extended areas adjacent to the tubule.

often formed concentrically arranged cisternae (fig. 5c). Structures, known as Charcot-Böttcher crystals, were often observed in these cells (fig. 5e). The basal lamina was made up of several layers (up to 6 laminae densae) and was strongly undulated (fig. 5e), probably as a result of tubules diameter reduction in the absence of spermatogenic elements.

At both ages, 24 and 36 months, the lamina propria of seminiferous tubules was made up of 1–2 layers of myofibroblasts and occasionally occurring fibroblasts (fig. 5f). No differences were found in comparison with the control animals of the same age. Numerous Leydig cells occupying extended areas adjacent to the tubules were present in the interstitium. Their number, location and morphology were identical in both TgHD and WT animals, regardless of age (fig. 5f).

An immunohistochemical cell proliferation assay detecting Ki67 and PCNA expression was performed in order to determine the mitotic activity of spermatogonia. Ki67 protein is expressed only in the nuclei of spermato-

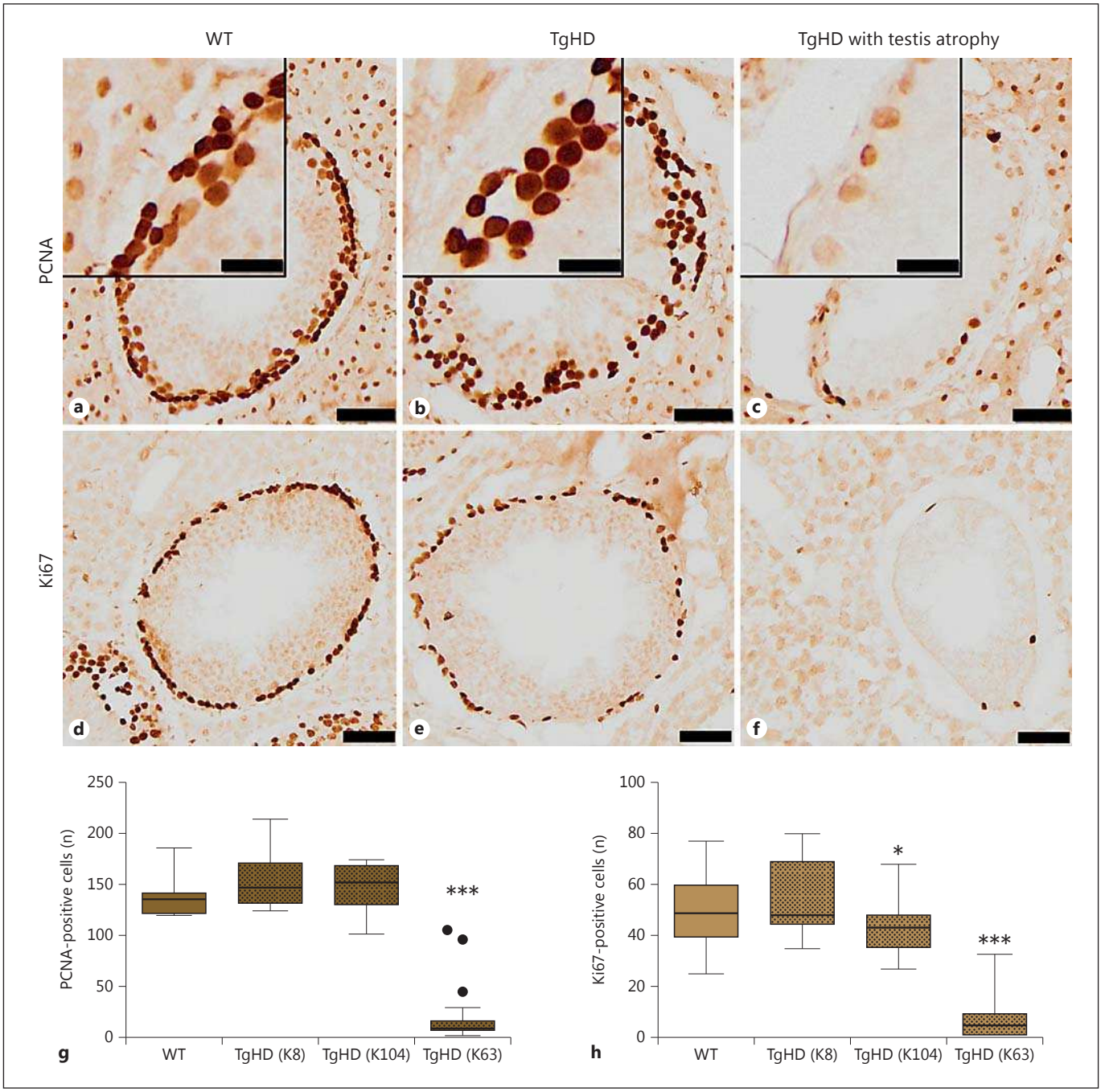


Fig. 6. Proliferative analysis. PCNA (**a–c**) and Ki67 (**d–f**) staining revealed decreased number of spermatocytes (**c**) and spermatogonia (**f**) in seminiferous epithelium of the TgHD boar (K63) at the age of 36 months. **g**, **h** The number of spermatogenic cells of the TgHD boar (K63) was significantly decreased compared with the WT boar ($p < 0.05$). Seminiferous tubules of the 24-month-old (**b**) and 36-month-old (**e**) transgenic boars showed similar staining of spermatogenic cells to WT (**a**, **d**). **h** The exception is testicular tissue from TgHD boar K104, in which spermatogonia (Ki67-positive cells) were determined in a significantly reduced number of seminiferous tubules compared with WT. Scale bars = 50 μm (20 μm in **insets**). Results were plotted as mean ± SD of positive cells per seminiferous tubule. $p < 0.05$ was considered significant. * $p = 0.0151$; *** $p < 0.0001$.

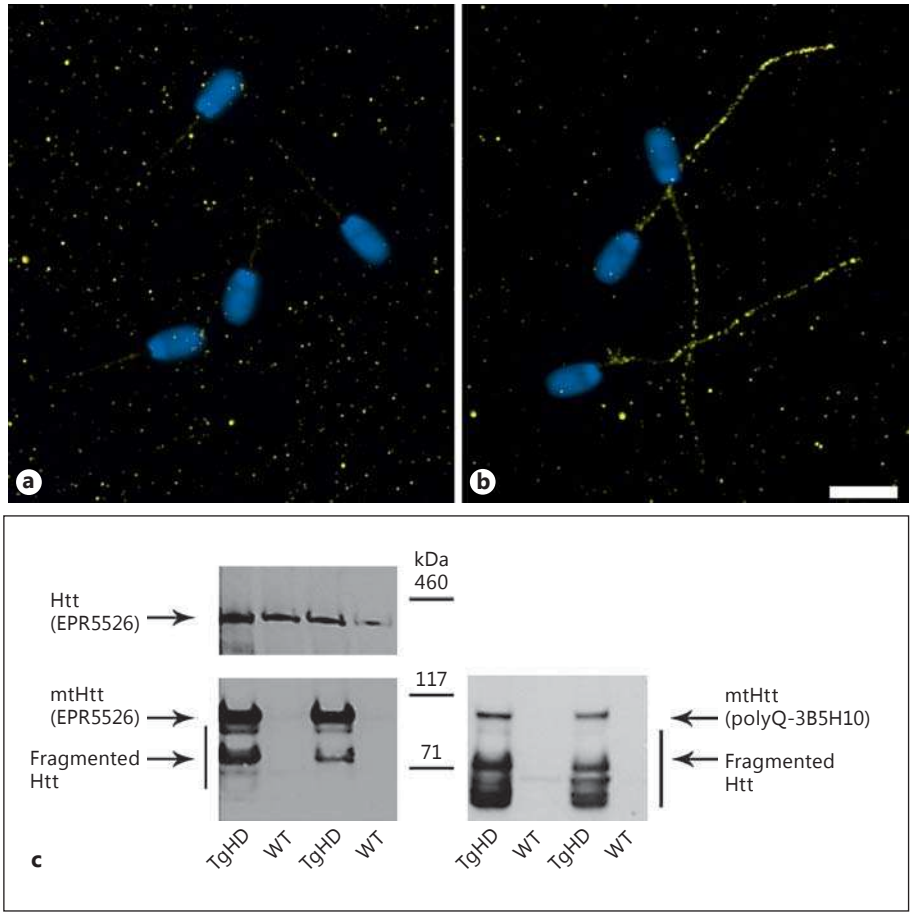
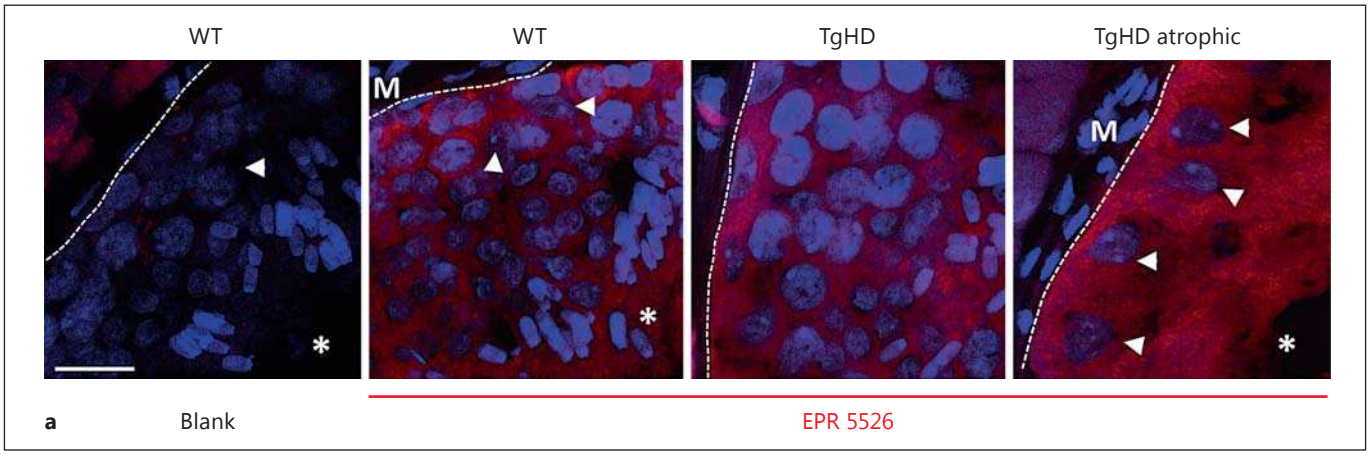


Fig. 7. Presence of Htt in spermatozoa cells. **a**, **b** Immunofluorescence dotted signal of the polyglutamine-containing proteins (3B5H10 antibody, yellow; color in online version only) was detected along whole spermatozoa tail of TgHD boars (**b**) but not in WT cells (**a**). Scale bar = 20 μm. **c** Western blot analysis detected endogenous (Htt) and transgenic (mtHtt) huntingtin by EPR5526 antibody, specific to N-terminal part of huntingtin. PolyQ antibody (3B5H10) as well as Htt antibody (EPR5526) detected fragments of mtHtt.

gonia, while PCNA occurs also in the nuclei of primary spermatocytes in normal seminiferous epithelium [25] – 90.86% (726/799) of seminiferous tubules lacked PCNA-positive cells and 90.08% (745/827) of seminiferous tubules contained no Ki67-positive cell in the testis of the 36-month-old TgHD boar (K63; fig. 6a–f). Spermatogonia serve as stem cells in the process of differentiation to spermatozoa, and their proliferative activity secures normal spermatogenesis. That means that impaired spermatogenesis involved 90% of seminiferous tubules. These results confirmed impaired spermatogenesis observed by EM (as indicated above). The remaining 10% of seminiferous tubules contained spermatogonia and spermatocytes stained by anti-PCNA and anti-Ki67 antibodies, but their number was significantly reduced in comparison with the seminiferous tubules of a WT boar. A significant decrease in the quantity of spermatogonia was also observed in the testis of the other TgHD boar at the age of 36 months (K104) in comparison with the WT one. It suggested that the spermatogonial proliferation was reduced in TgHD boars at the age of 36 months (n = 2, K104 and K63). No difference was found in the testes of the 24-month-old TgHD boar (K8; fig. 6g, h).

Sperm and Testicular Degeneration: The Direct Effect of mtHtt

Supposing that sperm pathology is caused by the toxic effect of mtHtt, experiments showing localization and expression of mtHtt were done. The presence of the polyglutamine-containing proteins was observed in structures in the spermatozoa tail of TgHD boars using 10-nm gold particles examined under EM (fig. 3f). This finding was confirmed by a dotted immunohistochemical signal detected along the whole spermatozoa tail in all tested ages of F1- and F2-generation TgHD boars (but not in WT controls) using 3B5H10 (anti-N-terminal fragment of 171 aa of human Htt with 65Q) antibody (fig. 7a). Western blot analysis using EPR5526 (anti-N-terminal fragment of Htt) confirmed highly abundant mtHtt and a slightly lower level of endogenous Htt in spermatozoa (fig. 7b). In ad-



Color version available online

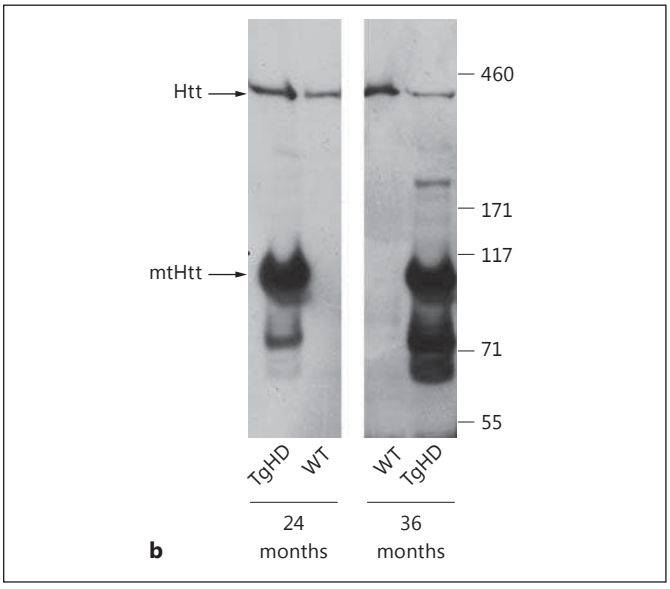


Fig. 8. Expression of Htt in testes. M = Myoid cells. **a** Total huntingtin protein was visualized immunohistochemically with EPR5526 antibody in sections of WT and transgenic (TgHD) porcine testis. EPR5526 (red; color in online version only) was detected in seminiferous epithelium demarcated by seminiferous tubule basement membrane (dashed line) and lumen (asterisk). Myoid cells were negatively stained. Cell nuclei were counterstained with DAPI (blue). Prominent Sertoli cell nuclei (arrowheads) present in atrophic seminiferous tubule (TgHD atrophic) documented the loss of germ cells. Blank, WT testis section stained only with A647-conjugated secondary antibody. Scale bar = 20 μm. **b** Western blot analysis of testis; representative sample shown. Anti-AB1 antibody detected endogenous Htt, transgenic mtHtt and fragments.

dition, anti-polyQ antibody (3B5H10) as well as anti-huntingtin antibody (EPR5526) revealed a fragmented form of mtHtt, which was reported to cause cellular toxicity [26]. Sperm samples from all tested ages in both generations (F1, F2) were analyzed, and no significant change in expression between samples was detected (only representative data from two pairs of samples are shown; fig. 7).

Consequently, we looked at Htt localization in TgHD and WT seminiferous tubules from testes of 24- and 36-month-old boars. The signal was widely spread in spermatogenic and Sertoli cells in normal as well as in the atrophic seminiferous tubules (fig. 8a). Western blot analysis showed a high expression of mtHtt form compared with endogenous Htt. The huntingtin antibody also revealed fragmented forms of mtHtt that may contribute to toxicity of the transgene (fig. 8b).

In order to eliminate the neuronal effect on the testicular phenotype, levels of fertility-related hormones were measured in the blood serum of two age groups: 7- to 17-month-old TgHD (n = 14) and WT (n = 9) boars, and 15–30-month-old TgHD (n = 11) and WT (n = 6) boars. Levels of testosterone, LH and inhibin-α were analyzed (see online suppl. material SM 2). No significant difference was observed between TgHD and WT animals.

Mapping the HIV1-HD-548aaHTT-145Q Transgene Integration

To detect any and all sites of vector integration into the pig genome in the transgenic lineage, we performed long-insert jumping library whole-genome sequencing of transgenic animals from F0 (founder female) and F2 gen-

erations in comparison with a negative control. This method, which involves sequencing the ends of genomic DNA fragments after circularization and size reduction, has previously been shown to be an effective platform for vector integration site discovery in transgenic sheep and mice [21]. Whole-genome sequencing of jumping libraries prepared from random fragments (mean size 3.6 kb) resulted in an average of 65.5× coverage of mapped inserts across each base in the haploid genome for all 3 animals. The paired-end reads were examined for the signature of vector integration into the genome: chimeric fragments consisting of pig genomic sequence on one end and a portion of the introduced vector/transgene on the other. No integrations were detected in the negative control genome, while a single identical vector integration was detected in each of the F0 and F2 generations of TgHD animals. As suggested previously from FISH analysis, the integration occurred into chromosome 1q [9]. The jumping library analysis revealed that 5.3 kb of the HIV1-HD-548aaHTT-145Q vector DNA integrated as expected via the HIV LTR sequences and harbored an intact HD-548aaHTT145Q expression cassette. Sanger sequencing of the junctions with genomic DNA showed that the integration was in reverse orientation relative to the genomic sequence, between chr1 228,641,631 and 228,641,637, with loss of the 5 intervening bases of genomic DNA. This integration does not directly disrupt any annotated gene, and no further breakpoint or integration complexity or other genomic rearrangement was apparent at the resolution of the jumping library sequencing.

Discussion

In this study we described sperm and testicular degeneration, which is a result of the presence of mtHtt protein in the testes of transgenic minipig boars expressing the N-terminal part of human mtHtt. Cohorts of TgHD and their WT controls of F1 and F2 generations were directly compared.

Previous studies on HD rodent models showed male sterility that was assumed to be due to a reduction of spermatozoa [15]. We confirmed this phenotype in a large animal model of HD, the minipig. Additionally, we reported both the reduction of spermatozoa and also their function measured by motility, progressivity and in vitro penetration assay. Spermatozoa of TgHD boars had severe problems to penetrate the minipig oocytes. The difference in values of sperm parameters was evident at 13 months and worsened with age. A comparison of animals from F1 and F2 generations of the same age (25–36 months) showed slightly worse values of all observed parameters in the F2 generation. There was also a wider variability of sperm parameters in the F2 generation, probably caused by a larger cohort of animals in the F2 group. Furthermore, EM analysis revealed deformation of the mitochondrial sheath in the tail midpiece of TgHD spermatozoa. Folded or coiled tails and a double or triple axoneme with fused mitochondrial sheaths were also observed. This can be caused by a failure of the disjunction of excess cytoplasm, which results in the presence of cytoplasmic droplets. This phenomenon, together with spermatozoa motility dysfunction, can be related to a decrease of mitochondrial energetic metabolism and functional impairment of respiratory chain complex II (unpublished observations). Occasionally, nucleus deformation associated with incomplete chromatin condensation and abnormal acrosome occurred in transgenic spermatozoa (but not in WT controls). These abnormalities were more pronounced in the F2 generation. Moreover, nearly all the spermatozoa of TgHD animals of the F2 generation contained a cytoplasmic droplet, and their ejaculate lacked residual bodies.

The testicular degeneration reported here is in agreement with observations in mice (R6/2 and Yac72) [14, 15], as well as in postmortem samples from humans [13]. Multinucleated spermatogenic cells were frequently present in the seminiferous epithelium of 24- and 36-month-old TgHD boars. The spermatogonia were shrunk and had dilated endoplasmic reticulum, swollen mitochondria and condensed chromatin in the nucleus. Spermatocytes and spermatids were observed occasionally. Reduced numbers of developing spermatocytes and spermatids were also observed in HD patients [13] and YAC128 mice [27]. Some tubules contained only Sertoli cells. Sertoli cells were characterized by increased density and vacuolization of the cytoplasm, dilatation of endoplasmic reticulum, structural alterations of the nuclei and swollen mitochondria. These are features typical for early or advanced apoptosis. Moreover, proliferative analysis of seminiferous tubules with elongated spermatozoa showed fewer cells expressing the proliferative markers PCNA and Ki67 in TgHD animals. The apoptotic nature of the cell death in a large number of degenerating spermatids with diffuse cytoplasmic vacuolization, condensed nuclei and electron-dense cytoplasm which were phagocytized and degraded by Sertoli cells was also observed in a mouse model lacking endogenous huntingtin YAC72$^{-/-}$ [14]. Similar-

ly, seminiferous tubules of YAC128 were disrupted by large vacuoles [27]. In addition, the seminiferous tubule wall was thickened in HD patients [13]. In the TgHD minipig, the thick basal lamina was made up of several layers (2–3 laminae densae) compared with the WT minipig. At the age of 36 months, spermatogenesis was more affected in comparison with the age of 24 months. Sertoli cells contained extreme amounts of endoplasmic reticulum and structures known as Charcot-Böttcher crystals. The basal lamina was made up of several layers (up to 6 laminae densae) and was strongly undulated, resulting probably from a reduction of the diameter of the tubules in the absence of spermatogenic elements. The rate of degenerative changes in the testicular samples of the 2 transgenic boars at the age of 36 months was different. The changes were less pronounced in boar K104 than in boar K63 and resembled those of the TgHD boar at the age 24 months. Boar K63 also showed more change in sperm parameters, including atrophy of seminiferous epithelium and impaired spermatogenesis. The difference in severity of pathology between age-matched boars might be a consequence of variation in the progression of the disease and genetic background of individual minipigs. The age of onset of HD depends on CAG length (around 70%), but also on other factors like polymorphism, modifier genes, etc. (30%) [28, 29]. We suggest that polymorphisms of proteins interacting with huntingtin could contribute to different degrees of testicular degeneration between transgenic boars of the same age.

After demonstrating testicular abnormalities we intended to clarify the reason for the pathological phenotype. We focused on the design of the transgenic minipig model. The number of CAG repeats was chosen in order to expect an earlier phenotype. This number of repeats imitates juvenile HD in patients. It has an earlier onset and faster progress, but the disease has the same characteristics as the adult form. Mixed CAG/CAA repeats, instead of just CAG, were used to increase the stability of the insert. This has been used in several rodent models (YAC128, BACHD mice) that also showed the phenotype [30, 31]. Therefore, the design was not a problem. We also checked whether the insertion of the lentiviral construct did not interrupt any coding sequence in the pig genome. Since the result was negative, the question was whether the defect in the testes was due to the expression of mtHtt or as a result of changed levels of fertility-related hormones.

There is evidence that the expression of mutant Htt leads to selective cellular dysfunction and degeneration [32]. The most affected cells are neurons. However, we also provided data for spermatozoa degeneration and testicular dysfunction in a minipig model of HD. We showed a high and stable expression of endogenous Htt as well as the transgenic N-truncated mutant form of human Htt in spermatozoa as well as in spermatogenic and Sertoli cells, and also in the atrophic seminiferous tubules of TgHD testes. In addition, we detected fragments of mtHtt in spermatozoa as well as in testes. It has been published that smaller fragments of mtHtt cause cellular toxicity and induce apoptosis [33, 34]. Furthermore, mutant N-terminal Htt fragments were also detected in tissues from HD patients and mouse models in the presymptomatic stage, suggesting their role in the progression of HD [35–37]. These facts also support our statement that the N-terminal part of human mtHtt causes testicular pathology in transgenic minipig boars.

Although testicular degeneration in HD is well described in mouse models (R6/2 and YAC128), it is not clear whether this phenotype is independent or a consequence of alterations of the hypothalamic-pituitary-gonadal axis (GnRH). A significant loss of GnRH neurons starting from 5 weeks of age followed by decreasing levels of plasma testosterone at 12 weeks of age was found in R6/2 mice [11], while testicular atrophy without concomitant loss of GnRH neurons was described in a YAC128 mouse model [13]. An analysis of testosterone levels in YAC128 mice did not reveal any significant difference compared with controls, even when testicular atrophy was already present [13]. Furthermore, testosterone treatment had no effect on the peripheral phenotype of HD, e.g. body weight loss or motor function in R6/2 mice [11]. An analysis of complete neuroendocrine status in HD patients showed no significant difference in the plasma levels of LH, FSH and testosterone between all male HD patients and controls [38]. However, Markianos et al. [39] observed significantly lower testosterone and LH levels in HD patients compared with healthy controls. These conflicting results suggested a detailed hormonal analysis of our porcine model. We observed no significant difference in the levels of fertility-related hormones between TgHD and control boars, and no changes in libido were observed during regular collection of semen. Moreover, in the interstitium, the number, location and morphology of the Leydig cells were identical regardless of the age or genotype of the animal, and no differences were found in the lamina propria of seminiferous tubules of the TgHD boars in both ages compared with WT controls. Similarly, unaffected Leydig cells were observed between degenerating tubules of stromal interstitial tissue in YAC72$^{-/-}$

mice [14]. Our results support the idea that testicular degeneration and fertility defects are related to mtHtt expression in testes and not to peripheral hormonal changes.

In conclusion, we demonstrated a failure in sperm parameters and extensive testicular pathology in a minipig model of HD. We showed that insertion of the lentiviral construct did not interrupt any coding sequence in the pig genome and suggest that the testicular defect was caused by the presence of mtHtt and its fragmented cytotoxic form in testicular tissue, since hormonal changes were not measured between TgHD boars and their WT controls.

Acknowledgments

This work was supported by the CHDI Foundation (A-5378), the project EXAM from the European Regional Development Fund (CZ.1.05/2.1.00/03.0124), RVO 67985904, the Norwegian Financial Mechanism 2009–2014, the Ministry of Education, Youth and Sports (project contract MSMT-28477/2014) and Charles University (grant SVV 260083), and National Sustainability Programme No. LO1609 (MEYS CR).

Disclosure Statement

One of the authors, Dr. David S. Howland is employed by the CHDI Foundation, which supported this work. Dr. Howland was not involved in designing the experiments or in writing this article, and did not make the decision to publish it. He was a consultant. The authors have no other conflicts of interest to declare.

References

1 Vonsattel JP, DiFiglia M: Huntington disease. J Neuropathol Exp Neurol 1998;57:369–384.
2 Stanek LM, Sardi SP, Mastis B, Richards AR, Treleaven CM, Taksir T, Misra K, Cheng SH, Shihabuddin LS: Silencing mutant huntingtin by adeno-associated virus-mediated RNA interference ameliorates disease manifestations in the YAC128 mouse model of Huntington's disease. Hum Gene Ther 2014;25:461–474.
3 Dufour BD, Smith CA, Clark RL, Walker TR, McBride JL: Intrajugular vein delivery of AAV9-RNAi prevents neuropathological changes and weight loss in Huntington's disease mice. Mol Ther 2014;22:797–810.
4 Squitieri F, Di Pardo A, Favellato M, Amico E, Maglione V, Frati L: Pridopidine, a dopamine stabilizer, improves motor performance and shows neuroprotective effects in Huntington disease R6/2 mouse model. J Cell Mol Med 2015;19:2540–2548.
5 Holm IE, Alstrup AK, Luo Y: Genetically modified pig models for neurodegenerative disorders. J Pathol 2016;238:267–287.
6 Dolezalova D, Hruska-Plochan M, Bjarkam CR, Sorensen JC, Cunningham M, Weingarten D, Ciacci JD, Juhas S, Juhasova J, Motlik J, Hefferan MP, Hazel T, Johe K, Carromeu C, Muotri A, Bui J, Strnadel J, Marsala M: Pig models of neurodegenerative disorders: utilization in cell replacement-based preclinical safety and efficacy studies. J Comp Neurol 2014;522:2784–2801.
7 Vodicka P, Smetana K Jr, Dvorankova B, Emerick T, Xu YZ, Ourednik J, Ourednik V, Motlik J: The miniature pig as an animal model in biomedical research. Ann NY Acad Sci 2005;1049:161–171.
8 Matsuyama N, Hadano S, Onoe K, Osuga H, Showguchi-Miyata J, Gondo Y, Ikeda JE: Identification and characterization of the miniature pig Huntington's disease gene homolog: evidence for conservation and polymorphism in the CAG triplet repeat. Genomics 2000;69:72–85.
9 Baxa M, Hruska-Plochan M, Juhas S, Vodicka P, Pavlok A, Juhasova J, Miyanohara A, Nejime T, Klima J, Macakova M, Marsala S, Weiss A, Kubickova S, Musilova P, Vrtel R, Sontag EM, Thompson LM, Schier J, Hansikova H, Howland DS, Cattaneo E, DiFiglia M, Marsala M, Motlik J: A transgenic minipig model of Huntington's disease. J Huntingtons Dis 2013;2:47–68.
10 Guo J, Zhu P, Wu C, Yu L, Zhao S, Gu X: In silico analysis indicates a similar gene expression pattern between human brain and testis. Cytogenet Genome Res 2003;103:58–62.
11 Papalexi E, Persson A, Bjorkqvist M, Petersen A, Woodman B, Bates GP, Sundler F, Mulder H, Brundin P, Popovic N: Reduction of GnRH and infertility in the R6/2 mouse model of Huntington's disease. Eur J Neurosci 2005;22:1541–1546.
12 Van Raamsdonk JM, Pearson J, Murphy Z, Hayden MR, Leavitt BR: Wild-type huntingtin ameliorates striatal neuronal atrophy but does not prevent other abnormalities in the YAC128 mouse model of Huntington disease. BMC Neurosci 2006;7:80.
13 Van Raamsdonk JM, Murphy Z, Selva DM, Hamidizadeh R, Pearson J, Petersen A, Bjorkqvist M, Muir C, Mackenzie IR, Hammond GL, Vogl AW, Hayden MR, Leavitt BR: Testicular degeneration in Huntington disease. Neurobiol Dis 2007;26:512–520.
14 Leavitt BR, Guttman JA, Hodgson JG, Kimel GH, Singaraja R, Vogl AW, Hayden MR: Wild-type huntingtin reduces the cellular toxicity of mutant huntingtin in vivo. American J Hum Genet 2001;68:313–324.
15 Sathasivam K, Hobbs C, Turmaine M, Mangiarini L, Mahal A, Bertaux F, Wanker EE, Doherty P, Davies SW, Bates GP: Formation of polyglutamine inclusions in non-CNS tissue. Hum Mol Genet 1999;8:813–822.
16 Hanscom C, Talkowski M: Design of large-insert jumping libraries for structural variant detection using Illumina sequencing. Curr Protoc Hum Genet 2014;80:7221–7229.
17 Li H, Durbin R: Fast and accurate long-read alignment with Burrows-Wheeler transform. Bioinformatics 2010;26:589–595.
18 Tarasov A, Vilella AJ, Cuppen E, Nijman IJ, Prins P: Sambamba: fast processing of NGS alignment formats. Bioinformatics 2015;31:2032–2034.
19 Li H, Handsaker B, Wysoker A, Fennell T, Ruan J, Homer N, Marth G, Abecasis G, Durbin R: The Sequence Alignment/Map format and SAMtools. Bioinformatics 2009;25:2078–2079.
20 Barnett DW, Garrison EK, Quinlan AR, Stromberg MP, Marth GT: BamTools: A c++ API and toolkit for analyzing and managing BAM files. Bioinformatics 2011;27:1691–1692.
21 Chiang C, Jacobsen JC, Ernst C, Hanscom C, Heilbut A, Blumenthal I, Mills RE, Kirby A, Lindgren AM, Rudiger SR, McLaughlan CJ, Bawden CS, Reid SJ, Faull RL, Snell RG, Hall IM, Shen Y, Ohsumi TK, Borowsky ML, Daly MJ, Lee C, Morton CC, MacDonald ME, Gusella JF, Talkowski ME: Complex reorganization and predominant non-homologous repair following chromosomal breakage in karyotypically balanced germline rearrangements and transgenic integration. Nat Genet 2012;44:390–397.
22 Brand H, Pillalamarri V, Collins RL, Eggert S, O'Dushlaine C, Braaten EB, Stone MR, Chambert K, Doty ND, Hanscom C, Rosenfeld JA, Ditmars H, Blais J, Mills R, Lee C, Gusella JF, McCarroll S, Smoller JW, Talkowski ME, Doyle AE: Cryptic and complex chromosomal aberrations in early-onset neuropsychiatric disorders. Am J Hum Genet 2014;95:454–461.

23 Talkowski ME, Rosenfeld JA, Blumenthal I, Pillalamarri V, Chiang C, Heilbut A, Ernst C, Hanscom C, Rossin E, Lindgren AM, Pereira S, Ruderfer D, Kirby A, Ripke S, Harris DJ, Lee JH, Ha K, Kim HG, Solomon BD, Gropman AL, Lucente D, Sims K, Ohsumi TK, Borowsky ML, Loranger S, Quade B, Lage K, Miles J, Wu BL, Shen Y, Neale B, Shaffer LG, Daly MJ, Morton CC, Gusella JF: Sequencing chromosomal abnormalities reveals neurodevelopmental loci that confer risk across diagnostic boundaries. Cell 2012;149:525–537.
24 Rausch T, Zichner T, Schlattl A, Stutz AM, Benes V, Korbel JO: Delly: Structural variant discovery by integrated paired-end and split-read analysis. Bioinformatics 2012;28:i333–i339.
25 Steger K, Aleithe I, Behre H, Bergmann M: The proliferation of spermatogonia in normal and pathological human seminiferous epithelium: an immunohistochemical study using monoclonal antibodies against Ki-67 protein and proliferating cell nuclear antigen. Mol Hum Reprod 1998;4:227–233.
26 Miller JP, Holcomb J, Al-Ramahi I, de Haro M, Gafni J, Zhang N, Kim E, Sanhueza M, Torcassi C, Kwak S, Botas J, Hughes RE, Ellerby LM: Matrix metalloproteinases are modifiers of huntingtin proteolysis and toxicity in Huntington's disease. Neuron 2010;67:199–212.
27 Van Raamsdonk JM, Pearson J, Rogers DA, Bissada N, Vogl AW, Hayden MR, Leavitt BR: Loss of wild-type huntingtin influences motor dysfunction and survival in the YAC128 mouse model of Huntington disease. Hum Mol Genet 2005;14:1379–1392.
28 Langbehn DR, Hayden MR, Paulsen JS: CAG-repeat length and the age of onset in Huntington disease (HD): a review and validation study of statistical approaches. Am J Med Genet B Neuropsychiatr Genet 2010;153B:397–408.
29 Tabrizi SJ, Reilmann R, Roos RA, Durr A, Leavitt B, Owen G, Jones R, Johnson H, Craufurd D, Hicks SL, Kennard C, Landwehrmeyer B, Stout JC, Borowsky B, Scahill RI, Frost C, Langbehn DR: Potential endpoints for clinical trials in premanifest and early Huntington's disease in the TRACK-HD study: analysis of 24-month observational data. Lancet Neurol 2012;11:42–53.
30 Gray M, Shirasaki DI, Cepeda C, Andre VM, Wilburn B, Lu XH, Tao J, Yamazaki I, Li SH, Sun YE, Li XJ, Levine MS, Yang XW: Full-length human mutant huntingtin with a stable polyglutamine repeat can elicit progressive and selective neuropathogenesis in BACHD mice. J Neurosci 2008;28:6182–6195.
31 Pouladi MA, Stanek LM, Xie Y, Franciosi S, Southwell AL, Deng Y, Butland S, Zhang W, Cheng SH, Shihabuddin LS, Hayden MR: Marked differences in neurochemistry and aggregates despite similar behavioural and neuropathological features of Huntington disease in the full-length BACHD and YAC128 mice. Hum Mol Genet 2012;21:2219–2232.
32 Bhide PG, Day M, Sapp E, Schwarz C, Sheth A, Kim J, Young AB, Penney J, Golden J, Aronin N, DiFiglia M: Expression of normal and mutant huntingtin in the developing brain. J Neurosci 1996;16:5523–5535.
33 Hackam AS, Singaraja R, Wellington CL, Metzler M, McCutcheon K, Zhang T, Kalchman M, Hayden MR: The influence of huntingtin protein size on nuclear localization and cellular toxicity. J Cell Biol 1998;141:1097–1105.
34 Martindale D, Hackam A, Wieczorek A, Ellerby L, Wellington C, McCutcheon K, Singaraja R, Kazemi-Esfarjani P, Devon R, Kim SU, Bredesen DE, Tufaro F, Hayden MR: Length of huntingtin and its polyglutamine tract influences localization and frequency of intracellular aggregates. Nat Genet 1998;18:150–154.
35 Mende-Mueller LM, Toneff T, Hwang SR, Chesselet MF, Hook VY: Tissue-specific proteolysis of huntingtin (Htt) in human brain: evidence of enhanced levels of N- and C-terminal Htt fragments in Huntington's disease striatum. J Neurosci 2001;21:1830–1837.
36 Wang CE, Tydlacka S, Orr AL, Yang SH, Graham RK, Hayden MR, Li S, Chan AW, Li XJ: Accumulation of N-terminal mutant huntingtin in mouse and monkey models implicated as a pathogenic mechanism in Huntington's disease. Hum Mol Genet 2008;17:2738–2751.
37 Wellington CL, Singaraja R, Ellerby L, Savill J, Roy S, Leavitt B, Cattaneo E, Hackam A, Sharp A, Thornberry N, Nicholson DW, Bredesen DE, Hayden MR: Inhibiting caspase cleavage of huntingtin reduces toxicity and aggregate formation in neuronal and nonneuronal cells. J Biol Chem 2000;275:19831–19838.
38 Saleh N, Moutereau S, Durr A, Krystkowiak P, Azulay JP, Tranchant C, Broussolle E, Morin F, Bachoud-Levi AC, Maison P: Neuroendocrine disturbances in Huntington's disease. PLoS One 2009;4:e4962.
39 Markianos M, Panas M, Kalfakis N, Vassilopoulos D: Plasma testosterone in male patients with Huntington's disease: relations to severity of illness and dementia. Ann Neurol 2005;57:520–525.

^{31}P MR Spectroscopy of the Testes and Immunohistochemical Analysis of Sperm of Transgenic Boars Carried N-terminal Part of Human Mutated Huntingtin

^{31}P MR spektroskopie varlat a imunohistochemická analýza spermií transgenních kanců nesoucích N-terminální část lidského mutovaného huntingtinu

Abstract

Huntington's disease (HD) is an inherited autosomal neurodegenerative disorder characterized by motor dysfunctions, behavioral and cognitive disturbances. It affects predominantly the brain, however, changes were found also in peripheral tissues. Some of these changes can result from direct expression of mutant huntingtin; its highest levels have been found in the brain and testes. In 2009 we established a minipig model of HD (TgHD) expressing N-terminal (548aa) part of human mutated huntingtin encoded 124 CAG/CAA repeats. Previous research has revealed the presence of reduced fertility and fewer spermatozoa per ejaculate in TgHD boars started at 13 months of age. The aim of this study was to determine changes in the testes of 24 months old transgenic boars (F2 generation *in vivo*) using non-invasive methodology of ^{31}P magnetic resonance (MR) spectroscopy as well as to perform imunohistochemical analysis of TgHD sperm collected from-form F1 and F3 generation before HD onset. The results have shown significant reduction of relative phosphodiester concentration in testicular parenchyma of TgHD boars compared to wild type (WT) ones of the same ages. Moreover immunohistochemical analysis of sperm collected from TgHD and WT have revealed exclusive anti-polyQ specific (clone 3B5H10) as well as significantly increased anti-huntingtin (clone EPR5526) staining in transgenic spermatozoa tails in comparison with WT counterparts. Thus, our results are suggestive of the negative impact of human mutated huntingtin on testes metabolism as well as sperm abnormalities.

http://dx.doi.org/10.14735/amcsnn20152S28

The authors declare they have no potential conflicts of interest concerning drugs, products, or services used in the study.
Autoři deklarují, že v souvislosti s předmětem studie nemají žádné komerční zájmy.
The Editorial Board declares that the manuscript met the ICMJE "uniform requirements" for biomedical papers.
Redakční rada potvrzuje, že rukopis práce splnil ICMJE kritéria pro publikace zasílané do biomedicínských časopisů.

M. Jozefovicova[1,2], V. Herynek[1], F. Jiru[1], M. Dezortova[1], J. Juhasova[3], S. Juhas[3], J. Klima[3], B. Bohuslavova[3], J. Motlik[3], M. Hajek[1]

[1] MR Unit, Department of Diagnostic and Interventional Radiology, Institute for Clinical and Experimental Medicine, Prague, Czech Republic
[2] Department of NMR Spectroscopy and Mass Spectroscopy, Faculty of Chemical and Food Technology, Slovak University of Technology, Bratislava, Slovak Republic
[3] Institute of Animal Physiology and Genetics, AS CR, v.v.i., Libechov, Czech Republic

✉
MVDr. Stefan Juhas, Ph.D.
Institute of Animal Physiology and Genetics
AS CR, v.v.i.
Rumburska 89, 277 21 Libechov
Czech Republic
e-mail: juhas@iapg.cas.cz

Accepted for review: 3. 10 2015
Accepted for print: 20. 10 2015

Key words

Huntington's disease – testes – sperm – minipig – magnetic resonance spectroscopy

Klíčová slova

Huntingtonova nemoc – varlata – spermie – miniprase – magnetická rezonanční spektroskopie

Souhrn

Huntingtonova nemoc (HN) je autozomálně dominantně dědičné neurodegenerativní onemocnění charakterizované motorickým deficitem, poruchami chování a kognitivních funkcí. Postihuje především mozek, přičemž změny související s HN byly nalezeny rovněž i v periferních tkáních. Některé z nich mohou být způsobeny přímou expresí mutovaného huntingtinu, jehož nejvyšší koncentrace byly nalezeny v mozku a varlatech pacientů s HN. V roce 2009 jsme vytvořili miniprasečí model HN (TgHD) exprimující N-terminální (548aa) část lidského mutovaného huntingtinu kódujícího 124 CAG/CAA repetic. Na základě předchozích experimentů byla u TgHD kanců od 13. měsíce věku zjištěna zhoršená schopnost reprodukce a snížený počet spermií v ejakulátu. Cílem této studie bylo prokázat změny ve varlatech 24 měsíčních transgenních kanců (F2 generace *in vivo*) pomocí neinvazivní metody ^{31}P magnetické rezonanční spektroskopie a provést imunohistochemickou analýzu TgHD spermií odebraných z F1 a F3 generace před projevením se klinických příznaků HN. Na základě vyšetření magnetickou rezonancí bylo zjištěno signifikantní snížení relativní koncentrace fosfodiesterů v testikulárním parenchymu TgHD kanců v porovnání s netransgenními jedinci (WT) stejné věkové kategorie. Rovněž imunohistochemická analýza spermií odebraných z TgHD a WT kanců odhalila výrazné anti-polyQ specifické (klon 3B5H10) stejně tak i signifikantně zvýšené anti-huntingtin (klon EPR5526) barvení v bičících transgenních spermiích v porovnání s netransgenními spermiemi. Na základě našich výsledků lze usuzovat, že lidský mutovaný huntingtin má negativní vliv na metabolizmus varlat a způsobuje zvýšený výskyt abnormalit spermií.

Aim/s of the study

Huntington's disease (HD) is a progressive neurodegenerative disorder caused by an unstable CAG (cytosine-adenine-guanine) trinucleotide expansion in the huntingtin gene (HTT) on the short arm of chromosome 4 [1,2]. Symptoms such as motor dysfunctions, behavioral and cognitive disturbances [3] have been early linked to neurodegeneration occurring in the brain [4]. However, abnormal changes were also found in peripheral tissues like testes, heart, pancreas, skeletal muscle [5]; some of these changes can result from direct expression of mutant huntingtin [4] or a loss of wild type one [6]. The highest levels of huntingtin expression have been found in the brain and testes [4]. Indeed, the human brain and testes have the most similar gene expression pattern in compare with other organ types [7]. It has been also shown that men with HD have reduced numbers of germ cells (spermatocytes, spermatids) and abnormal seminiferous tubule morphology in the testis [8]. In addition mutant huntingtin not only compromised spermatogenesis in HD patients but also in R6/2 [9] and YAC128 [8] mouse models. Infertility in the R6/2 males was due either to death of GnRH neurons or to a reduction in GnRH expression leading to a downstream impairment of the gonadotropic hormones [9]. On the other side the testes atrophy and decline in sperm production and final azoospermia in R6/2 mice occurs at the time of onset of neuropathological symptoms and all of them were sterile by eight weeks of age [5]. In the YAC128 mouse model, testicular degeneration develops prior to 12 months of age, but at 12 months, there is no evidence for decreased testosterone levels or loss of GnRH neurons in the hypothalamus [8]. As we have reported previously, HD porcine model shows lower spermatozoa counts compared to wild type animals [10]. In addition motility and oocyte penetration capability of sperm is reduced in transgenic boars bearing mutant huntingtin [11] and indicates defects in its spermatogenesis. The mechanism of mutant huntingtin action on cell functions is not completely known as it interacts with a plethora proteins and localizes to various cellular structures like the nucleus, Golgi apparatus, mitochondria etc. [12–16]. Recently mutant huntingtin has been shown to bind to the basal body and to interfere with cilliogenesis [17–19]. Thus insight into pathogenesis occurring in the testis and spermatogenesis of men and animal models of HD may reveal common critical pathways which lead to degeneration in the brain and other organs.

^{31}P MR spectroscopy is a non-invasive method that can be used to monitor the metabolite changes of different tissue types like the brain, liver, muscle and testes *in vivo* [20–25]. The *in vivo* ^{31}P MR spectrum of the testis contains seven characteristic peaks originating from phosphomonoesters (PME), phosphodiesters (PDE), adenosine triphosphate (three peaks representing three phosphates ATP-α, -β, -γ), inorganic phosphate (Pi), and phosphocreatine (PCr), which is not present in the testicle, but arises from a muscle contamination [23]. In the spectrum can be also identified small signal of nicotinamide adenine dinucleotide (NADH). PME peak represents mainly phosphocholine and phosphoethanolamine and is directly related to the rate of phospholipid biosynthesis. The PDE peak reflects phospholipid breakdown and mostly consists of glycerophosphocholine and glycerophosphoethanolamine. Inorganic phosphate is a catabolic product of metabolites containing phosphates [25–27]. Moreover ^{31}P MR spectroscopy of the human testes may differentiate between normal testes, oligozoospermia, azoospermia that is caused by epididymal obstructions in the reproductive tract (normal spermatogenesis) and azoospermia that is caused by testicular failure (no germ cells are present) [23].

The minipig boars have huge testis, what allowed big voxel size in this area. In addition the minipig males also produce a large volume of sperm as an optimal biological material collected by noninvasive approach.

The aim of this study was to determine changes in the testes and sperm of transgenic minipigs expressing human mutated huntingtin related to testicular abnormalities by using ^{31}P MR spectroscopy and immunohistochemistry.

Methods

Minipigs

Transgenic boars (TgHD) according to [10] were obtained from The Institute of Animal Physiology and Genetics (Libechov, Czech Republic). The minipig strain resulted from Minnesota, Gottingen, and domestic farm strain cross-breeding and their successful germ line transmission occurred through successive F0, F1, F2 and F3 generations. Until now several phenotypes like the reduced male reproductive parameters (e. g. fewer spermatozoa per ejaculate), impaired mitochondrial function in spermatozoa [11], lower level of total creatine in the brain [28] and blood serum cytokine imbalance [29] have been detected. The founder sow, born July 2009, as well as the offsprings are without clinical symptoms of HD at the present time. We expect the outbreak of the clinical symptoms in the second half of their life, i.e., after the 10th year. Transgenic boars

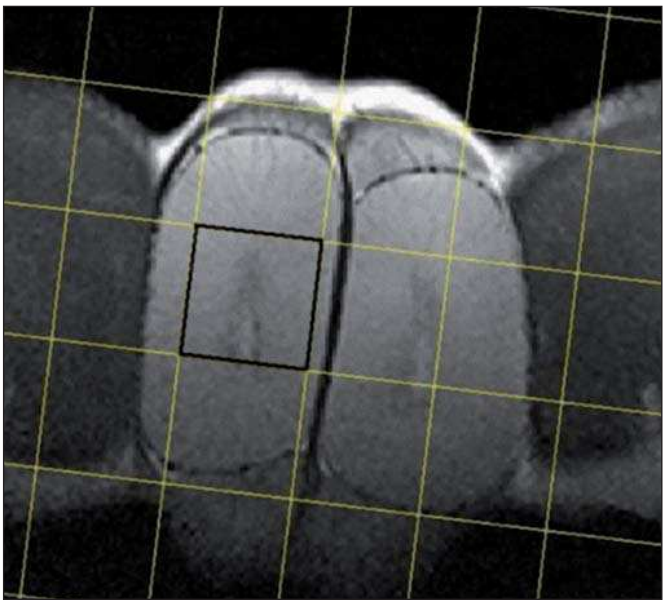


Fig. 1. The positioning of the spectroscopic grid in the program jSIPRO.

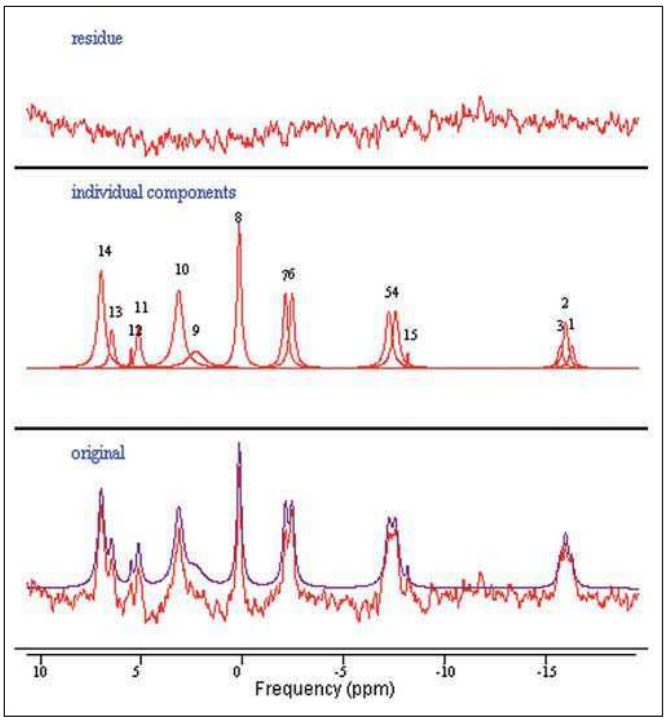


Fig. 2. Quantification of representative spectrum from one voxel in testis in the program AMARES.

Teoretical fitted spectrum (middle) obtained by a quantification of the experimental spectrum (below) is denoted with a blue color. Upper image shows residual.

from F2 generation (2 years old) before HD onset (n = 5) and control wild type (WT) siblings (n = 5) were used for MR spectrometry study. For sperm IHC study we used transgenic and WT boars from F1 (n = 2; 4–5 years old) and F3 (n = 2; 2 years old).

In MR spectrometry study minipigs were anesthetized with propofolum (Propofol 1% Fresenius) in combination with intramuscular TKX mixture (Tiletaminum 5 mg/kg, Zolazepamum 5 mg/kg, Ketaminum 5 mg/kg, Xylazine 1 mg/kg). For premedication of minipigs TKX mixture in combination with diazepamum (0.25 mg/kg, Apaurin) was used. A pulse oximeter clipped to a tail for continuous control of pulse rate and oxygen level in the blood of the minipig was used. Semen from transgenic and nontransgenic animals was collected without anesthesia using phantom dummy.

All experiments were conducted with the approval of the State Veterinary Administration of the Czech Republic and in accordance with Czech regulations and guidelines for animal welfare.

MR spectrometry experiments

All MR experiments were performed using a whole body 3T MR scanner (Siemens Magnetom Trio). A flexible dual tuned ^{1}H/^{31}P surface coil (Rapid Biomedical, Rimpar, Germany) was placed beneath the animal in a prone position. For spectra localization, three perpendicular images obtained by a standard T2W turbo-spin echo sequence (TR = 4 400 ms, TE = = 99 ms, slice thickness = 4 mm), were used. ^{31}P spectra were acquired by 2D-CSI (Two-Dimensional Chemical Shift Imaging) sequence (TR = 4 000 ms, TE = 2 ms, VOI = 240 × 240 × 25 mm, matrix size 8 × 8 × 1, voxel size 30 × 30 × 25 mm, NA = 8). The positioning of the voxel in 2D-CSI spectroscopic grid is shown in Fig. 1. An automatic magnetic field homogeneity adjustment based on B0 mapping supplemented by manual shimming was used.

MR spectrometry post-processing

Data were preprocessed using a program jSIPRO [30] which involved k-space Hamming filtering and shifting of the spectroscopic grid for the best fit positioning of the selected voxel in the area of the testis. The single spectrum from the chosen voxel was exported as a text file and then processed in the jMRUI software package (version 5.0). Processing involved manual phase correction (zero and first-order), hard phase of all signals and setting of the reference (PCr) peak to 0 ppm. Spectra were analyzed using the AMARES algorithm [31], which is a part of jMRUI software package [32]. Eight signals were evaluated in each spectrum. PCr and NADH peaks were fitted as singlets, Pi, PME, PDE as two singlet peaks (with restricted linewidths to maximal value 100 Hz), α-ATP, γ-ATP peaks as doublets, and β-ATP as a triplet. Signal of α-ATP, β-ATP and γ-ATP peaks were not constraint to each other because the integrals of α-ATP, γ-ATP signals might also contain contribution of adenosine diphosphate (ADP). For all signals a Lorentzian shape was used.

The spectra quantification was performed without fixed values of zero and first-order phase by using weighting function (quarter-sine wave) in an interval 1–10 and truncation of first 2 points of the FID.

The relative metabolite concentrations were calculated as ratios to γ-ATP. We are aware that the γ-ATP signal may also include a contribution from ADP. However, although β-ATP signal would be unambiguous, it might be affected by the partial excitation due to its large chemical shift displacement, hence it is not suitable as a reference [22,33]. The testicular spectra from minipigs were contaminated by the signals from muscle, as evidenced by the presence of PCr signal in the spectra. Therefore the ATP and Pi concentrations were corrected by deduction of the muscle contribution calculated from the PCr signal. Muscle ATP (20% of the PCr signal) and Pi (10% of the PCr signal) contribution was determined from the spectra in the

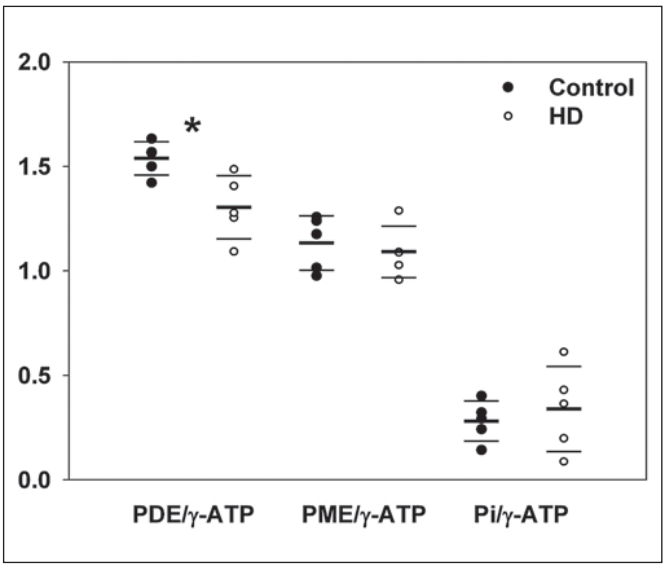


Fig. 3. Distribution of the ratios PDE/γ-ATP, PME/γ-ATP and Pi/γ--ATP in the group of TgHD minipigs (○) and control wild type (nontransgenic) siblings (•).

Horizontal lines show average values and standard deviations. Asterix marks significance level $p < 0.05$.

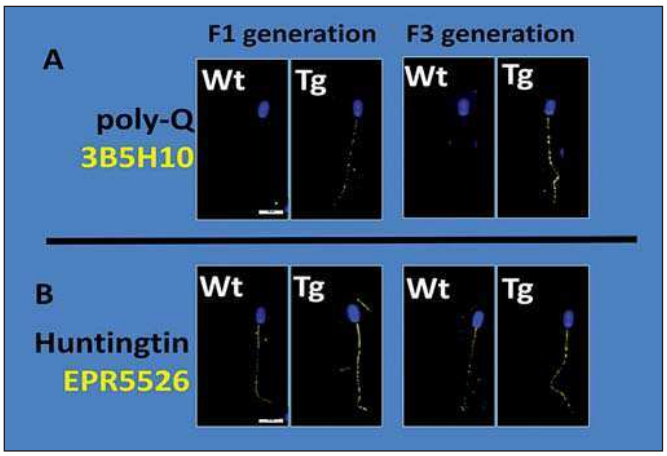


Fig. 4. Immunofluorescence staining of transgenic (Tg) and control (Wt) boar spermatozoa (F1 and F3 generation).

Fig. 4A) Using 3B5H10 antibody, no poly-Q specific signal was found in WT spermatozoa. Distinct 3B5H10 positive signal (yellow) was detected solely in transgenic HD spermatozoa.

Fig. 4B) Anti-huntingtin specific antibody EPR5526 revealed huntingtin epitopes either in WT and Tg spermatozoa of both generations. The punctuate staining was more intensive in Tg than in WT spermatozoa. Sperm heads were counterstained with DAPI (blue). Scale bar represents 10 μm.

muscle obtained during the same measurement (in two WT minipigs) and was in agreement with published data [24,34]. Concentrations of PME and PDE were not corrected as their concentrations in the muscle are negligible with respect to the experimental error.

IHC

The semen of F1 and F3 generation boars (both genotypes) was collected using the hand-glove technique. After three times washing in BTS buffer spermatozoa were diluted to 5×10^5 concentration and cytospinned onto a microscopy slide and air dried. The prepared spermatozoa were permeabilized using 0.04% Triton in PBS for 5 min. 30 min blocking step was carried out by blocking buffer consisting of permeablization buffer supplemented with 0.2% non-fat dry milk. Anti-huntingtin (1 : 400, clone EPR5526, MABN1105, Millipore) or anti-polyQ antibody (1 : 1000, clone 3B5H10, P1874, Sigma Aldrich) were applied in blocking solution for 1 hour at room temperature. Excess of antibody was removed by three times washing in washing buffer (0.05% Tween in PBS). Goat anti-mouse or anti-rabbit secondary antibodies conjugated with Alexa 555 (1 : 500, A-21422, A-21428, Invitrogen) were used for 1 hour incubation to visualize bound primary antibodies. After three times washing stained spermatozoa were mounted in DAPI containing mounting medium and evaluated under a virtual slide system VS120-FL Olympus (Olympus, Czech Republic).

Statistics

One spectrum from each testis was obtained; i.e., two spectra from each minipig (5 TgHD and 5 WT) were evaluated. The relative concentrations (γ-ATP, e.g. PDE/γ-ATP, PME/γ-ATP and Pi/γ-ATP) were tested for normal distribution (Shapiro-Wilk test) and homogeneity of variance (Levene's tests – absolute and squared deviations). These conditions were fulfilled for all these ratios and therefore were evaluated using two-tailed Student's t-test. A level $p < 0.05$ was considered as a statistically significant difference. The value of NADH was not evaluated due to its small concentration in the spectra.

For statistical analysis of EPR5526 fluorescently stained spermatozoa an unpaired t-test and Mann-Whitney nonparametric test were employed using GraphPad PRISM software (GraphPad Software, San Diego, CA, USA).

Results

MR spectrometry

The quantification of a typical spectrum from one testis using the program AMARES is shown in Fig. 2.

The ratios PDE/γ-ATP, PME/γ-ATP and Pi/γ-ATP of the WT and TgHD minipigs are displayed in Fig. 3. Distribution within the experimental groups and average values including standard deviations are also presented. We found significant decrease in the ratio PDE/γ-ATP ($p = 0.022$) in TgHD animals compared to WT and no difference in relative PME and Pi concentrations.

IHC

Ejaculated boar spermatozoa were specifically stained with anti-polyQ specific antibody clone 3B5H10. F1 and F3 generation of transgenic spermatozoa showed punctuate fluorescence signal along the whole sperm tail, whereas there was no 3B5H10 signal in tails of wild type (Fig. 4A) spermatozoa. Contrary to expectation 3B5H10 staining was missing in mitochondrion of both genotypes. EPR5526 antibody raised against N-terminal part of huntingtin protein showed similar punctuates staining in both genotypes (Fig. 4B). However, the fluorescence signal was stronger in transgenic spermatozoa and resulted into statistical significance (Fig. 5).

Conclusion

HD is a neurodegenerative disorder that besides the brain affects also peripheral organs like the testes. However it is still unclear whether these abnormal changes are result

from direct toxicity of the mutant huntingtin in the peripheral organ or they are a consequence of the brain damage [8,35].

TgHD minipig fertility problems manifested themselves in the reduced male reproductive parameters; nevertheless the minipigs were up to now without typical clinical manifestations (uncontrolled muscle movements, weight loss, etc.) of HD.

Determination of changes in the testes has been mainly based on analysis of seminal fluid or level of hormone concentrations so far [27], because biopsy is invasive and may cause additional damage to spermatogenesis [23]. ^{31}P MR spectrometry is a non-invasive technique that can be used to monitoring the testicular function as shown in several studies [23–26]. Importantly, this paper represents the first ^{31}P CSI spectroscopy of HD large animal model testes.

For the measurement of human or rats testes ^{31}P non-localized or localized single volume spectroscopy were used. Multivoxel spectroscopy might fail due to anatomical conditions of the testes [23,24,26,36]. However, anatomical size of minipig's testes allows multivoxel measurements with relatively big voxel size in this area (30 × × 30 × 25 mm), therefore we used spatially resolved multivoxel spectroscopic method Chemical Shift Imaging (CSI).

In earlier studies spectra were quantified by simple integration of individual peaks [25,26]. We used a program AMARES instead, which is commonly used for analysis of ^{31}P MR spectrometry spectra.

Previous ^{31}P MR spectrometry studies of the human testes demonstrated that the PME/ATP ratio is a more sensitive parameter to monitor testicular function, i.e., to differentiate between normal testes, oligozoospermia and azoospermia, than the PDE/ATP or Pi/ATP ratio [24,27]. However, we have not found a significant change in PME/ATP ratio in our study with HD minipigs. Nevertheless we should admit that small numbers of the animals in WT and TgHD groups was a limiting factor in this study.

It has been shown that PDE peak mainly consists of glycerophosphocholine (GPC) and in smaller amount of glycerophosphoethanolamine (GPE) [25]. GPC is present in very high concentrations in seminal fluid and a positive correlation was found between the GPC concentration and sperm motility [25,37]. Therefore we suggest that a decreased level of PDE/γ-ATP ratio in TgHD minipigs may be related to decreased concentration of seminal fluid or the changes in sperm motility. Similar reduction in the testicular phosphodiesters (PDE) PDE/ATP ratio ($p < 0.05$) as we have observed in our TgHD minipig study has been detected in rats that were pair-fed for 10 weeks on ethanol containing liquid diet (36% ethanol of total calories) [38].

The aim of sperm IHC study was to focus on the localization of huntingtin protein in spermatozoa as the sperm flagellum is a modified primary cilium and also contains mitochondria. Using poly-Q specific staining we could clearly discriminate transgenic and wild type spermatozoa respectively. Huntingtin specific staining localized endogenous porcine huntingtin as well as mutant N-terminal part of human huntingtin to the tail (flagellum) of the porcine sperm. Both staining protocols showed punctate staining along the midpiece and principal piece. This indicates huntingtin protein localization to either axonemal structures or outer dense fibers. Our data cannot support huntingtin localization to mitochondria.

Finally we studied transgenic minipigs (F1, F2 and F3 generation) expressing mutant huntingtin without clinical symptoms of HD at the time of the study. Until now several phenotypes like the reduced male reproductive parameters were detected which manifest themselves by decrease of relative PDE concentration in the testes revealed by ^{31}P MR spectroscopy. We hypothesize that the PDE decrease was related to decreased concentration of seminal fluid or the changes in sperm motility. These findings were in agreement with immunofluorescent detection of N-terminal fragment of human huntingtin in tails of spermatozoa collected from TgHD boars.

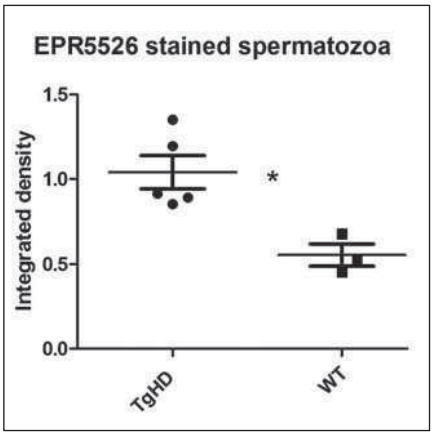


Fig. 5. Quantification of fluorescent signal in TgHD (n = 5) and WT (n = 3) boars´ spermatozoa (F1 and F3 generation).

Integrated densities of five randomly selected spermatozoa from each animal (TgHD and WT) were measured by image processing program ImageJ (Rasband, W.S., U. S. National Institutes of Health, Bethesda, Maryland, USA). Asterisk marks significance level $p < 0.05$.

Acknowledgements

This work was supported by the MH CZ-DRO („Institute for Clinical and Experimental Medicine – IKEM, IN 00023001"), Program Research and Development for Innovation Ministry of Education, Youth and Sports ExAM CZ.1.05/2.1.00/03.0124, Norwegian Financial Mechanism 2009–2014 and the Ministry of Education, Youth and Sports under Project Contract no. MSMT-28477/2014 "HUNTINGTON" 7F14308, CHDI Foundation (A-5378, A-8248) and RVO: 67985904.

References

1. van den Bogaard SJ, Dumas EM, Teeuwisse WM, Kan HE, Webb A, Roos RA et al. Exploratory 7-Tesla magnetic resonance spectroscopy in Huntington's disease provides in vivo evidence for impaired energy metabolism. J Neurol 2011; 258(12): 2230–2239. doi: 10.1007/s00415-011-6099-5.

2. van Oostrom JC, Sijens PE, Roos RA, Leenders KL. 1H magnetic resonance spectroscopy in preclinical Huntington's disease. Brain Res 2007; 1168: 67–71.

3. Bano D, Zanetti F, Mende Y, Nicotera P. Neurodegenerative processes in Huntington's disease. Cell Death Dis 2011; 2: e228. doi: 10.1038/cddis.2011.112.

4. van der Burg JM, Bjorkqvist M, Brundin P. Beyond the brain: widespread pathology in Huntington's disease. Lancet Neurol 2009; 8(8): 765–774. doi: 10.1016/S1474-4422(09)70178-4.

5. Sathasivam K, Hobbs C, Turmaine M, Mangiarini L, Mahal A, Bertaux F et al. Formation of polyglutamine inclusions in non-CNS tissue. Hum Mol Genet 1999; 8(5): 813–822.

6. Dragatsis I, Levine MS, Zeitlin S. Inactivation of Hdh in the brain and testis results in progressive neurodegeneration and sterility in mice. Nat Genet 2000; 26(3): 300–306.

7. Guo J, Zhu P, Wu C, Yu L, Zhao S, Gu X. In silico analysis indicates a similar gene expression pattern between human brain and testis. Cytogenetic Genome Res 2003; 103(1–2): 58–62.

8. Van Raamsdonk JM, Murphy Z, Selva DM, Hamidizadeh R, Pearson J, Petersen A et al. Testicular degeneration in Huntington's disease. Neurobiol Dis 2007; 26(3): 512–520.

9. Papalexi E, Persson A, Bjorkqvist M, Petersen A, Woodman B, Bates GP et al. Reduction of GnRH and infertility in the R6/2 mouse model of Huntington's disease. Eur J Neurosci 2005; 22(6): 1541–1546.

10. Baxa M, Hruska-Plochan M, Juhas S, Vodicka P, Pavlok A, Juhasova J et al. A transgenic minipig model of Huntington's disease. J Huntingtons Dis 2013; 2(1): 47–68. doi: 10.3233/JHD-130001.

11. Macakova M, Hansikova H, Antonin P, Hajkova Z, Sadkova J, Juhas S et al. Reproductive parameters and mitochondrial function in spermatozoa of F1 and F2 minipig boars transgenic for N-terminal part of the human mutated huntingtin. J Neurol Neurosurg Psychiatry 2012; 83: A16–A16.

12. del Toro D, Alberch J, Lazaro-Dieguez F, Martin-Ibanez R, Xifro X, Egea G et al. Mutant huntingtin im-

pairs post-Golgi trafficking to lysosomes by delocalizing optineurin/Rab8 complex from the Golgi apparatus. Mol Biol Cell 2009; 20(5): 1478–1492.
13. Oliveira JM, Jekabsons MB, Chen S, Lin A, Rego AC, Goncalves J et al. Mitochondrial dysfunction in Huntington's disease: the bioenergetics of isolated and in situ mitochondria from transgenic mice. J Neurochem 2007; 101(1): 241–249.
14. Enokido Y, Tamura T, Ito H, Arumughan A, Komuro A, Shiwaku H et al. Mutant huntingtin impairs Ku70-mediated DNA repair. J Cell Biol 2010; 189(3): 425–443. doi: 10.1083/jcb.200905138.
15. Jeon GS, Kim KY, Hwang YJ, Jung MK, An S, Ouchi M et al. Deregulation of BRCA1 leads to impaired spatiotemporal dynamics of gamma-H2AX and DNA damage responses in Huntington's disease. Mol Neurobiol 2012; 45(3): 550–563. doi: 10.1007/s12035-012-8274-9.
16. Chiu FL, Lin JT, Chuang CY, Chien T, Chen CM, Chen KH et al. Elucidating the role of the A2A adenosine receptor in neurodegeneration using neurons derived from Huntington's disease iPSCs. Hum Mol Genet 2015; pii: ddv318.
17. Keryer G, Pineda JR, Liot G, Kim J, Dietrich P, Benstaali C et al. Ciliogenesis is regulated by a huntingtin-HAP1-PCM1 pathway and is altered in Huntington's disease. J Clin Invest 2011; 121(11): 4372–4382. doi: 10.1172/JCI57552.
18. Kaliszewski M, Knott AB, Bossy-Wetzel E. Primary cilia and autophagic dysfunction in Huntington's disease. Cell Death Differ 2015; 22(9): 1413–1424. doi: 10.1038/cdd.2015.80.
19. Haremaki T, Deglincerti A, Brivanlou AH. Huntingtin is required for ciliogenesis and neurogenesis during early Xenopus development. Dev Biol 2015; pii: S0012-1606(15)30057-9. doi: 10.1016/j.ydbio.2015.07.013.
20. Bogner W, Chmelik M, Schmid AI, Moser E, Trattnig S, Gruber S. Assessment of (31)P relaxation times in the human calf muscle: a comparison between 3 T and 7 T in vivo. Magn Reson Med 2009; 62(3): 574–582. doi: 10.1002/mrm.22057.
21. Novak J, Wilson M, Macpherson L, Arvanitis TN, Davies NP, Peet AC. Clinical protocols for 31P MRS of the brain and their use in evaluating optic pathway gliomas in children. Eur J Radiol 2014; 83(2): e106–e112. doi: 10.1016/j.ejrad.2013.11.009.
22. Schmid AI, Chmelik M, Szendroedi J, Krssak M, Brehm A, Moser E et al. Quantitative ATP synthesis in human liver measured by localized 31P spectroscopy using the magnetization transfer experiment. NMR Biomed 2008; 21(5): 437–443.
23. van der Grond J, Laven JS, Lock MT, te Velde ER, Mali WP. 31P magnetic resonance spectroscopy for diagnosing abnormal testicular function. Fertil Steril 1991; 56(6): 1136–1142.
24. van der Grond J, Laven JS, te Velde ER, Mali WP. Abnormal testicular function: potential of P-31 MR spectroscopy in diagnosis. Radiology 1991; 179(2): 433–436.
25. van der Grond J, Laven JS, van Echteld CJ, Dijkstra G, Grootegoed JA, de Rooij DG et al. The progression of spermatogenesis in the developing rat testis followed by 31P MR spectroscopy. Magn Reson Med 1992; 23(2): 264–274.
26. Srinivas M, Degaonkar M, Chandrasekharam VV, Gupta DK, Hemal AK, Shariff A et al. Potential of MRI and 31P MRS in the evaluation of experimental testicular trauma. Urology 2002; 59(6): 969–972.
27. van der Grond J. Diagnosing testicular function using 31P magnetic resonance spectroscopy: a current review. Hum Reprod Update 1995; 1(3): 276–283.
28. Jozefovicova M, Herynek V, Jiru F, Dezortova M, Juhasova J, Juhas S et al. Minipig model of Huntington's disease: 1H magnetic resonance spectroscopy of the brain. Physiol Res 2015: in press.
29. Benova I, Skalnikova HK, Klima J, Juhas S, Motlik J. Activation of cytokine production in F1 and F2 generation of miniature pigs transgenic for N-terminal part of mutated human huntingtin. J Neurol Neurosurg Psychiatry 2012; 83: A16.
30. Jiru F, Skoch A, Wagnerova D, Dezortova M, Hajek M. jSIPRO – analysis tool for magnetic resonance spectroscopic imaging. Comput Methods Programs Biomed 2013; 112(1): 173–188. doi: 10.1016/j.cmpb.2013.06.018.
31. Vanhamme L, van den Boogaart A, Van Huffel S. Improved method for accurate and efficient quantification of MRS data with use of prior knowledge. J Magn Reson 1997; 129(1): 35–43.
32. Naressi A, Couturier C, Castang I, de Beer R, Graveron-Demilly D. Java-based graphical user interface for MRUI, a software package for quantitation of in vivo/medical magnetic resonance spectroscopy signals. Comput Biol Med 2001; 31(4): 269–286.
33. Valkovic L, Bogner W, Gajdosik M, Povazan M, Kukurova IJ, Krssak M et al. One-dimensional image-selected in vivo spectroscopy localized phosphorus saturation transfer at 7T. Magn Reson Med 2014; 72(6): 1509–1515. doi: 10.1002/mrm.25058.
34. Kemp GJ, Meyerspeer M, Moser E. Absolute quantification of phosphorus metabolite concentrations in human muscle in vivo by 31P MRS: a quantitative review. NMR Biomed 2007; 20(6): 555–565.
35. Hannan AJ, Ransome MI. Deficits in spermatogenesis but not neurogenesis are alleviated by chronic testosterone therapy in R6/1 Huntington's disease mice. J Neuroendocrinol 2012; 24(2): 341–356. doi: 10.1111/j.1365-2826.2011.02238.x.
36. van der Grond J, Van Pelt AM, van Echteld CJ, Dijkstra G, Grootegoed JA, de Rooij DG, et al. Characterization of the testicular cell types present in the rat by in vivo 31P magnetic resonance spectroscopy. Biol Reprod 1991;45(1):122–127.
37. Hinton BT, Setchell BP. Concentrations of glycerophosphocholine, phosphocholine and free inorganic phosphate in the luminal fluid of the rat testis and epididymis. J Reprod Fertil 1980; 58(2): 401–406.
38. Farghali H, Williams DS, Gavaler J, Van Thiel DH. Effect of short-term ethanol feeding on rat testes as assessed by 31P NMR spectroscopy, 1H NMR imaging, and biochemical methods. Alcohol Clin Exp Res 1991; 15(6): 1018–1023.

Original Paper

Neurodegener Dis 2017;17:213–226
DOI: 10.1159/000475467

Received after revision: November 8, 2016
Accepted: April 3, 2017
Published online: June 21, 2017

Mitochondrial Metabolism in a Large-Animal Model of Huntington Disease: The Hunt for Biomarkers in the Spermatozoa of Presymptomatic Minipigs

Jana Krizova[a] Hana Stufkova[a] Marie Rodinova[a] Monika Macakova[b] Bozena Bohuslavova[b] Daniela Vidinska[b] Jiri Klima[b] Zdenka Ellederova[b] Antonin Pavlok[b] David S. Howland[c] Jiri Zeman[a] Jan Motlik[b] Hana Hansikova[a]

[a]Laboratory for Study of Mitochondrial Disorders, Department of Pediatrics and Adolescent Medicine, General University Hospital and First Faculty of Medicine, Charles University, Prague, and [b]Laboratory of Cell Regeneration and Plasticity, Institute of Animal Physiology and Genetics, v.v.i., CAS, Libechov, Czech Republic; [c]CHDI Foundation, Princeton, NJ, USA

Keywords
Huntington disease · Large-animal model · Mutant huntingtin · Spermatozoa · Mitochondrial metabolism

Abstract
Background: Huntington disease (HD) is a fatal neurodegenerative disorder involving reduced muscle coordination, mental and behavioral changes, and testicular degeneration. In order to further clarify the decreased fertility and penetration ability of the spermatozoa of transgenic HD minipig boars (TgHD), we applied a set of mitochondrial metabolism (MM) parameter measurements to this promising biological material, which can be collected noninvasively in longitudinal studies. ***Objective:*** We aimed to optimize methods for MM measurements in spermatozoa and to establish possible biomarkers of HD in TgHD spermatozoa expressing the N-terminal part of mutated human huntingtin. ***Methods:*** Semen samples from 12 TgHD and wild-type animals, aged 12–65 months, were obtained repeatedly during the study. Respiration was measured by polarography, MM was assessed by the detection of oxidation of radiolabeled substrates (mitochondrial energy-generating system; MEGS), and the content of the oxidative phosphorylation system subunits was detected by Western blot. Three possibly interfering factors were statistically analyzed: the effect of HD, generation and aging. ***Results:*** We found 5 MM parameters which were significantly diminished in TgHD spermatozoa and propose 3 specific MEGS incubations and complex I-dependent respiration as potential biomarkers of HD in TgHD spermatozoa. ***Conclusions:*** Our results suggest a link between the gain of toxic function of mutated huntingtin in TgHD spermatozoa and the observed MM and/or glycolytic impairment. We determined 4 biomarkers useful for HD phenotyping and experimental therapy monitoring studies in TgHD minipigs.

KARGER

E-Mail karger@karger.com
www.karger.com/ndd

Dr. Hana Hansikova
Laboratory for Study of Mitochondrial Disorders, Department of Pediatrics and Adolescent Medicine, General University Hospital and First Faculty of Medicine
Charles University, Ke Karlovu 2, CZ–128 08 Prague 2 (Czech Republic)
E-Mail Hana.Hansikova@lf1.cuni.cz

Introduction

Huntington disease (HD) is a fatal neurodegenerative disorder with autosomal dominant inheritance affecting the central nervous system, leading to a lack of coordination, emotional problems, and also a loss of cognitive abilities in affected patients. The prevalence rate is about 1 in 15,000. The function of the causative protein, huntingtin (Htt; *HTT* gene), remains highly debated, but several studies predict its role in embryonic development and vesicular transport [1–6]. The *HTT* gene coding for the mutated Htt (mtHtt) carries a CAG (or CAA) triplet repetition expansion, which is translated to a polyglutamine tract (polyQ), prone to aggregate formation with cytotoxic effects, especially in striatal neurons. Patients with an abnormally increased number of CAG repeats (more than 36 in mtHtt) are vulnerable to being affected, whereas having more than 40 CAG repeats generally means a full penetrance of HD [7].

Despite its ubiquitous distribution in the brain and peripheral tissues, the expression of mtHtt is particularly detrimental to medium spiny neurons within the striatum. The gain of toxic function in mtHtt leads to a transcriptional dysregulation [8], synaptic dysfunction and excitotoxicity [9, 10], and defects in intracellular transport [11]. Moreover, mitochondrial dysfunction has been associated with HD pathogenesis [12–14]. Postmitotic neurons are particularly vulnerable to mitochondrial dysfunction because of their energetic dependence on oxidative phosphorylation (OXPHOS) [15, 16]. Previous studies have shown disturbances of mitochondrial metabolism (MM) in the HD brain, skeletal muscle, or even cultivated skin fibroblasts [17].

Htt is widely expressed in mammalian cells; apart from the brain it is also present in various tissues, including the testes [18]. Indeed, a study of 17 human tissues and a subsequent clustering analysis have shown that gene expression patterns in testes generally share the highest similarity with those in the brain [19]. Also, this neural and testicular expression of mtHtt also predominates in transgenic minipigs [20]. Intriguingly, reproductive disturbances were described both in human HD patients [21] as well as in transgenic (Tg) HD animal models, including our TgHD minipigs [20–23]. A decline in fertility in F1 generation TgHD boars caused by a reduced sperm count and penetration rate was first noticed at the age of 13 months and it remained constant until 26 months [24]. This decline in sperm count, motility, progressivity, and in vitro penetration parameters was unrelated to levels of fertility-related hormones or libido [20]. The number of TgHD spermatozoa that successfully penetrated the oocytes with an intact zona pellucida was significantly lower than that of the wild-type (WT) spermatozoa, indicating that the penetration activity of spermatozoa in TgHD boars was impaired [24]. After removing the zona pellucida enzymatically, penetration rates in the WT and TgHD groups were equal, suggesting that the presence of mtHtt interferes with the penetration of TgHD spermatozoa through the zona pellucida. Nevertheless, it remained unclear whether this interference might only be a result of the overall ultrastructural deformities of TgHD spermatozoa, or whether it is connected with any metabolic defect.

There is an ongoing debate about whether the spermatozoal energy metabolism relies on OXPHOS, glycolysis, or both. Glycolysis seems to be crucial for sperm motility [25]. However, capacitation, the process important for the penetration of zona pellucida, is characterized by a hyperactive motility caused by the increased amplitude of flagellar beats and higher ATP production, which is driven by OXPHOS in mitochondria [26]. Therefore, because the energetic metabolism of mammalian sperm is essential for sperm motility and fertilization [27], we decided to assess selected parameters of MM in the spermatozoa of TgHD minipig boars.

The aims of this study were therefore: (i) to assess the MM of TgHD spermatozoa, optimizing the methods used in the diagnostics of mitochondrial disorders, and (ii) to establish possible biomarkers of HD or its progression in TgHD spermatozoa.

Materials and Methods

Material

We used transgenic minipigs with 1 copy of the human *HTT* transgene. This transgene encoding the HD human promoter and the first 548 amino acids, including 124 glutamines (CAG/CAA), is integrated into chromosome 1 q24-q25 [24]. Semen samples were obtained from minipig boars (n = 12) from 2 generations (F1 and F2) aged between 12 and 65 months. The samples were obtained repeatedly during the 3-year course of the study up to 8 times per individual, according to the sample collection plan (Table 1). In these samples, the MM was assessed (including the tricarboxylic acid cycle [TCA], OXPHOS, and pyruvate dehydrogenase complex [PDHc]) by a detection of radiolabeled-substrates oxidation (mitochondrial energy-generating system; MEGS), and the content of OXPHOS subunits was detected by Western blot. Eight boars were selected for time-course measurements of spermatozoal respiration (Table 2). For each experiment, TgHD samples were obtained in a pair with those from WT siblings or very close relatives, which were used as the paired age-related controls. All components of this study were carried out in accordance with the Animal Care and Use Committee of the Institute of Animal Physiology and Genetics and

Table 1. Sample collection plan for MEGS capacity analysis

Pair No.	HD status	Generation	Age at the time of measurement, months							
1	TgHD	F1	12	14	19	23	30	36	42	47
1	WT	F1	22	24	29	33	41	46	53	58
2	TgHD	F1	12	14	19	23	30	36	42	47
2[a]	WT	F1	–[a]	–[a]	–[a]	40	48	53	60	65
3	TgHD	F2	14	18	22	23	30	34		
3	WT	F2	14	18	22	23	30	34		
4[b]	TgHD	F2	–[b]	22	25	32	36			
4	WT	F2	20	22	25	32	36			
5	TgHD	F2	16							
5	WT	F2	16							
6	TgHD	F2	16							
7	WT	F1	10	12						

Age is shown in completed months. HD, Huntington disease.

[a] The original WT boar in pair No. 2 died due to reasons unrelated to HD, so it was replaced by an older but a very close WT relative from the 4th measurement.

[b] At the age of 20 months, measurement of the TgHD boar in pair No. 4 was unsuccessful and therefore excluded from the data analysis.

were conducted according to current Czech regulations and guidelines for animal welfare and with the approval of the State Veterinary Administration of the Czech Republic.

Methods

Semen Collection and Spermatozoa Isolation

All ejaculates were evaluated using a sperm cell analyzer (Microptic, Barcelona, Spain) immediately after collection and the presence of mtHtt in spermatozoa of TgHD boars was examined [20]. Ejaculates were cooled to 25°C by being kept at room temperature (RT) for 30 min, then gently centrifuged for 20 min at 25°C and 250 *g*. The sperm-rich fraction of fresh ejaculate was diluted 2:1 with BST medium (0.2 M glucose, 20 mM sodium citrate, 14 mM sodium bicarbonate, 4 mM EDTA, 21 mM sodium chloride, 0.4% [w/V] kanamycin) and gently centrifuged for 20 min at 25°C and 250 *g*. Cells were washed two more times at a ratio of 1:2 and 1:1 with BST and this suspension was used for the analyses. Functional measurements (respiration and MEGS analyses) were performed during a single day. During shipping, the washed cells were incubated in ambient RT for respiration measurement. For the MEGS capacity analysis, spermatozoa were transported and stored on wet ice, and for protein/Western blot analyses cells were frozen immediately in liquid nitrogen and stored at –80°C until use. Standard spermiogram analysis, including motility and penetration test, was performed in each sample as described elsewhere [20].

Immunofluorescence Staining of Ejaculated Boar Spermatozoa

Spermatozoa were spun onto slides using cytospin (800 *g*, 5 min), air dried and stored frozen at –18°C. The staining protocol started with antigen retrieval in Tris-EDTA buffer (10 mM Tris, 1 mM EDTA solution, pH 9.0) at 80°C for 45 min. Slides were then immersed in permeabilization buffer for 5 min at RT, followed by a blocking step in blocking solution (0.2% low-fat dried milk in permeabilization buffer) for 30 min at RT. A mixture of primary antibodies (anti-polyglutamine antibody, clone 3B5H10, 1:1,000, P1874; anti-acetylated tubulin, 1:1,000, T7451; both Sigma-Aldrich, Saint Louis, MO, USA; anti-MTCO1, 1:500, ab14705; Abcam, Cambridge, UK) in blocking solution were applied on spermatozoa for 1 h at RT. After 3 washes in 0.05% (V/V) Tween 20 PBS, secondary antibodies (Alexa Fluor® 647 anti-mouse IgG1, A21240; Alexa Fluor® 555 anti-mouse IgG2a, A21137; Alexa Fluor® 488 anti-mouse IgG2b, A21141; all from ThermoFischer Scientific, Waltham, MA, USA) were added to the blocking solution and overlaid the cytospun spermatozoa. A 30-min incubation was followed by 3 washes in 0.05% (V/V) Tween 20 PBS and the slides were mounted in a DAPI mounting medium. Fluorescence was visualized using a Leica TCS SP5 confocal microscope (Leica Microsystems, Wetzlar, Germany) and image processing was performed using ImageJ software.

Table 2. Sample collection plan for respiration analysis

Pair No.	HD status	Generation	Age at the time of measurement, months			
1	TgHD	F1	30	36	42	47
1	WT	F1	41	46	53	58
2	TgHD	F1	30	36	42	47
2	WT	F1	48	53	60	65
3	TgHD	F2	20	23	30	34
3	WT	F2	20	23	30	34
4	TgHD	F2	20	25	32	36
4	WT	F2	20	25	32	36

Age is shown in completed months. HD, Huntington disease.

Table 3. *p* values for all validated effects (HD, generation, age) of MEGS and respirometric parameters

Parameter	Effect		
	HD	generation	age
MEGS			
1 – [1-^{14}C]pyruvate+malate	0.231	0.068	0.058
2 – [1-^{14}C]pyruvate+carnitine	0.019*	0.011*	0.013*
3 – [1-^{14}C]pyruvate+malate (–ADP)	0.348	0.019*	0.017*
4 – [1-^{14}C]pyruvate+malate+CCCP (–ADP)	–	0.011*	0.000*
4a – [1-^{14}C]pyruvate	0.030*	0.079	0.267
5 – [1-^{14}C]pyruvate+malate+atractyloside	0.157	0.013*	0.004*
6 – [U-^{14}C]malate+pyruvate+malonate	0.005*	0.131	0.070
7 – [U-^{14}C]malate+acetylcarnitine+malonate	0.082	0.187	0.000*
8 – [U-^{14}C]malate+acetylcarnitine+arsenite	0.348	–	0.012*
9 – [1,4-^{14}C]succinate+acetylcarnitine	0.058	0.370	0.390
Enzyme activity			
CS	0.381	0.968	–
MEGS/CS			
1/CS	–	0.126	0.079
2/CS	0.267	0.095	0.158
3/CS	–	0.004*	0.003*
4/CS	–	0.018*	0.001*
4a/CS	0.254	–	–
5/CS	0.390	0.018*	0.013*
6/CS	0.021*	0.558	0.131
7/CS	0.285	0.919	0.002*
8/CS	0.568	–	0.005*
9/CS	0.091	–	0.207
MEGS ratios			
(1/3)	–	–	–
(2/1)	–	–	–
(4/1)	–	0.030*	0.000*
(3/5)	0.278	–	0.417
(6/1)	0.267	0.282	0.000*
(7/6)	–	–	0.581
(7/8)	0.273	0.397	0.609
Respiratory states			
GM-rot	0.260	0.348	0.348
GMDc-rot (CI)	0.017*	0.095	0.348
S-AmA (CII)	0.440	–	–
aT-Z (CIV)	0.348	–	–
aTF-Z (CIV uncoupled)	0.126	–	–
Respiratory ratios			
CI/CII	–	–	–
CI/CIV	0.282	0.325	–
CII/CIV	–	–	–

MEGS analysis measure production of $^{14}CO_2$ formed by oxidation of radiolabeled substrate in the incubation containing specified substrates. All MEGS incubations were supplemented with ADP unless stated as –ADP. HD, Huntington disease. MEGS, mitochondrial energy-generating system capacity; CS, citrate synthase activity. * $p < 0.05$ was considered significant. Dash marks (–) indicate $p = 1.00$. Respiratory states measured in digitonin-permeabilized spermatozoa in KCl medium with: GM-rot, glutamate + malate (background corrected by rotenone inhibition); complex I-dependent respiration: GMDc-rot (CI), glutamate + malate + ADP + cytochrome *c* (corrected background by rotenone inhibition); complex II-dependent respiration: S-AmA (CII), succinate with glutamate + malate + ADP + cytochrome *c* and rotenone present in the chamber (corrected background by antimycin A inhibition); aT-Z, CIV maximal respiration with ascorbate and TMPD as electron carriers (autoxidation background corrected by sodium azide); aTF-Z (CIV), CIV uncoupled maximal respiration with ascorbate and TMPD as electron carriers and FCCP uncoupler (autoxidation background corrected by sodium azide).

Measurement of the MEGS Capacity

A homogenate from the fresh spermatozoa (total count between 6 and 32 × 10^9 sperm cells) was prepared at 4°C in a glass Potter-Elvehjem homogenizer in 150 mM KCl, 50 mM Tris-HCl, 2 mM EDTA, pH 7.4, with the addition of the 1% (V/V) protease inhibitor cocktail (Sigma-Aldrich). The homogenate was sonicated 3 times for 5 s by an ultrasonic homogenizer and used for MEGS analyses.

The MEGS capacity was analyzed in a fresh homogenate by measuring the oxidation rates of [1-^{14}C]pyruvate, [U-^{14}C]malate, and [1,4-^{14}C]succinate according to Janssen et al. [28], who published a detailed description of the theory of this method, in 10 different incubations (Table 3). For each reaction, 5 µL of homogenate with a protein concentration of 4–8 mg/mL was used. The $^{14}CO_2$ production was counted in Beckman Coulter LS (Beckman Coulter Inc., Brea, CA, USA). Rates of individual reactions were normalized to the protein concentration or to the control enzyme citrate synthase (CS) activity. The incubation ratios were compared to evaluate OXPHOS activities, the TCA cycle, and PDHc function. The total protein content was determined according to Lowry et al. [29].

Respirometry

Oxygen consumption was measured in digitonin-permeabilized spermatozoa at 37°C using an Oroboros Oxygraph-2k (Oroboros Instruments Corp., Innsbruck, Austria) in a 2-mL chamber with KCl-based medium (80 mM KCl, 10 mM Tris, 3 mM $MgCl_2$, 1 mM EDTA, 5 mM potassium phosphate; pH 7.4). The permeabilization of plasma membrane was realized by manual titration of approximately 4.1 µM digitonin (0.2 µL steps); the following protocol included multiple substrates and inhibitors as described previously [30, 31]: 10 mM glutamate, 2.5 mM malate, 1 mM ADP + Mg^{2+}, 0.5 µM rotenone, 10 mM succinate, 1 µM antimycin A, 4 mM ascorbate, and 0.4 mM TMPD (N,N,N′,N′-tetramethyl-1,4-phenylenediamine). Respiration was uncoupled by titration of 1.5 µM FCCP [carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone] and finally inhibited by the addition of 10 mM of sodium azide.

SDS-PAGE and Immunodetection of Mitochondrial Proteins

Spermatozoa cells were lysed in RIPA buffer (50 mM Tris-HCl [pH 7.4], 150 mM sodium chloride, 1 mM PMSF, 1 mM EDTA, 1% Triton X-100 and 0.1% [w/V] SDS, 1% [V/V] protease inhibitor cocktail; Sigma-Aldrich) for 20 min on ice and centrifuged at 51,000 *g* for 25 min at 4°C. Samples were dissociated in sample buffer containing 50 mM Tris-HCl (pH 6.8), 12% (V/V) glycerol, 4% SDS, 2% (V/V) 2-mercaptoethanol, and 0.01% (w/V) bromophenol blue for 30 min at 37°C. Proteins were separated on 12% polyacrylamide, 0.1% (w/V) SDS gels under standard tricine SDS-PAGE conditions. Ten micrograms of protein was loaded per lane. To check the linearity of the immunodetection signal, both 5 µg (50%) and 10 µg (100%) of protein (WT) were loaded per lane.

Proteins were electroblotted on Immobilon®-P PVDF membranes (Millipore, Billerica, MA, USA) using semi-dry transfer for 1 h at a constant current (0.7 mA/cm^2). Air-dried membranes (overnight) were rinsed in 100% methanol and blocked by 5% nonfat dried milk solution in Tris-buffered saline (TBS) for 2 h. Blots were incubated with primary antibodies in 0.1% (V/V) Tween 20 and 1% (w/V) nonfat dried milk solution in TBS for 2 h. For MM homeostasis, we used the following monitoring antibodies: anti-SDHA (ab14715), anti-SDHB (ab14714), anti-UQCR1 (ab110252), anti-NDUFA9 (ab14713), anti-NDUFB6 (ab110244), PDH Western blot antibody cocktail (ab110416), anti-MTCO1 (ab14705), anti-F1-α (ab110273), anti-F_O-OSCP (ab110276), anti-aconitase 2 (ab110321; all of the above from Abcam), anti-OPA1 (612606; BD Biosciences, San Jose, CA, USA) and anti-β-tubulin (T4026; Sigma-Aldrich). Detection by secondary antibody was carried out with anti-mouse or anti-rabbit IgG peroxidase-conjugated antibody (A8924, A0454; both from Sigma-Aldrich) in 0.1% Tween 20 and 1% nonfat dried milk solution in TBS for 1 h. Visualization was accomplished by SuperSignal West Femto Maximum Sensitivity Substrate (ThermoFisher Scientific, Waltham, MA, USA) on a SynGene imaging system (Synoptics Group, Cambridge, UK).

SDS-PAGE and Immunodetection of Htt

The frozen tissue samples of spermatozoa were lysed in RIPA buffer (150 mM NaCl, 0.05% NP-40, 1% sodium deoxycholate, 0.1% [w/V] SDS, 1% Triton X-100, 5 mM EDTA pH 8.0, 50 mM Tris-HCl pH 8.0, inhibitors of phosphatases and proteases) with 8 M urea and 1 mM DTT vortexed for at least 60 min at 4°C, then sonicated for 15 min and centrifuged at 15,000 *g* for 10 min at 4°C. Samples were loaded onto 3–8% Tris-acetate gel (ThermoFisher Scientific) and run at 150 V. The gel was transferred onto nitrocellulose membrane (ThermoFisher Scientific) at 250 mA for 45 min. Membranes were blocked in 5% nonfat dried milk for 1 h, and probed overnight with an appropriate antibody. For our experiments we used the anti-Htt antibody (1:3,000, EPR5526; Abcam). A peroxidase-conjugated secondary antibody (1:10,000, No. 711-035-152, anti-rabbit IgG; Jackson ImmunoResearch, West Grove, PA, USA) was used. A light reaction was induced by ECL (GE Healthcare, Chicago, IL, USA) and the signal was captured on CL-XPosure films (ThermoFisher Scientific). The exposed CL-XPosure films were scanned using a calibrated densitometer GS-800 and bands were quantified using Quantity One software (both Bio-Rad, Hercules, CA, USA) measuring the trace quantity.

Spectrophotometric Enzyme Activity Measurements

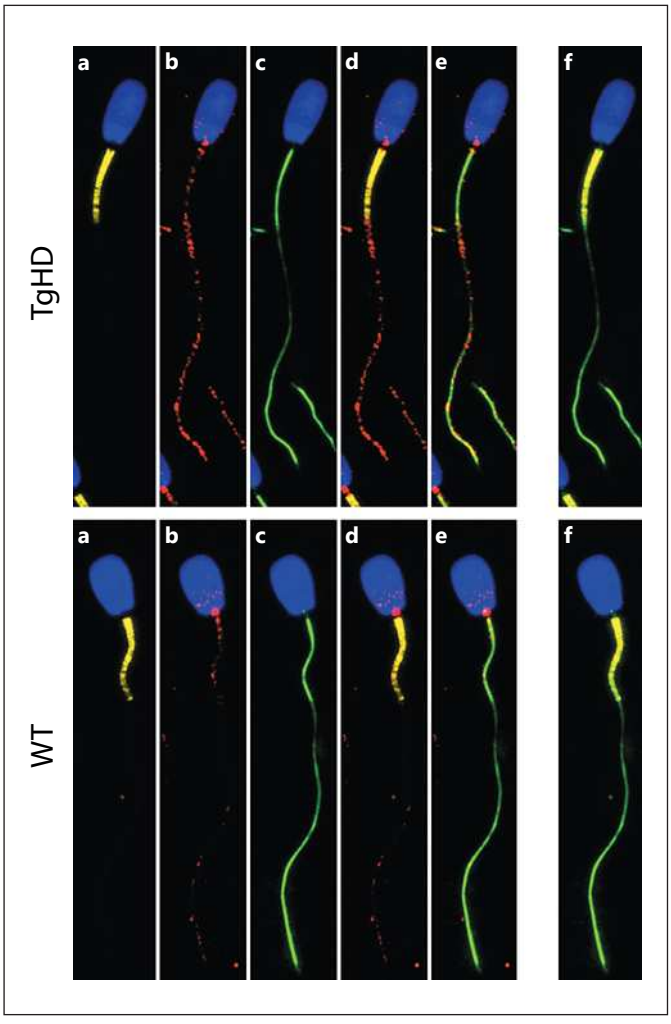
The activity of CS was measured spectrophotometrically in spermatozoal homogenates as described by Rustin et al. [32].

Histochemical Staining in Blue-Native Polyacrylamide Gels

Sixty micrograms of solubilized protein per lane was resolved by Blue-Native PAGE (BN-PAGE, gradient polyacrylamide 6–13% gel) as described elsewhere [33], stained for catalytic activity of OXPHOS complexes by histochemistry [34].

Statistical Analysis

In our set of minipig spermatozoa, we concentrated on the statistical analysis of 3 possibly interfering factors: (a) the effect of HD itself, (b) the effect of generation, and (c) the effect of aging (Table 3). The differences between observed enzyme activities/ratios in TgHD and WT controls with respect to age and generation were examined by linear regression models with mixed effects. The WT and corresponding age-matched TgHD pairs were considered as a random effect. Box-Cox transformations were used to gain normality of residuals. p values <0.05 (adjusted for multiple comparisons using the Benjamini and Yekutieli method) were considered statistically significant. Analyses were conducted using R statistical package, version 3.2.2.



Color version available online

Fig. 1. Localization of mitochondria and proteins containing polyglutamine-stretch (polyQ-proteins, including mtHtt) in spermatozoa from TgHD boars and WT controls. **a** Midpiece containing mitochondria is stained with anti-MTCO1 antibody (yellow). **b** Anti-polyglutamines (polyQ), clone 3B5H10, antibody (red) binding is prominent in TgHD spermatozoa. The polyQ signal in WT spermatozoa was near the background level. **c** Microtubules are stained with anti-acetylated tubulin (AcTub) antibody (green). **d** Merged signals for mitochondrial MTCO1 and polyQ. **e** Merged signals for AcTub and polyQ. polyQ was localized along the whole AcTub positive tail. **f** Merged signals for mitochondria and AcTub. $n_{TgHD} = 2$, $n_{WT} = 2$; polyQ signal only was stained in $n_{TgHD} = 6$, $n_{WT} = 6$. Magnification: ×1,200.

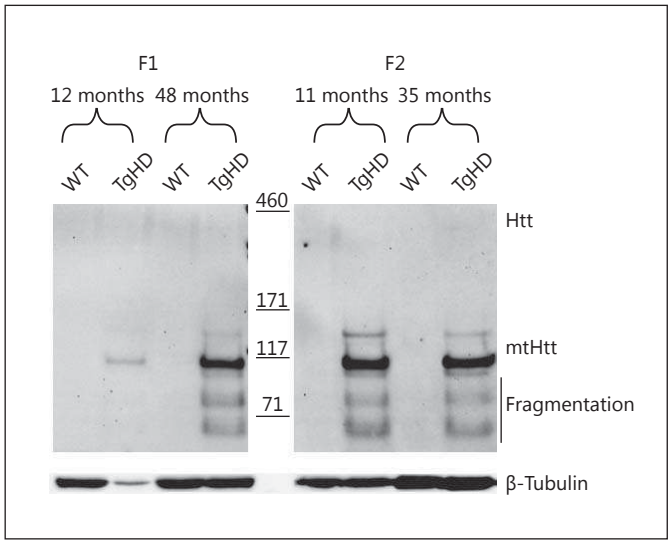


Fig. 2. High abundance and fragmentation of mtHtt compared to endogenous Htt levels in spermatozoa from TgHD boars of F1 and F2 generations at 2 different ages (from 12- and 48-, and 11- and 35-month-old boars, respectively). mtHtt and Htt was detected by anti-Htt antibody. The presence of mtHtt fragments was analyzed in $n_{TgHD} = 6$, $n_{WT} = 6$. A representative figure is shown.

Results

In this study we aimed to optimize the methods for MM measurements in spermatozoa and to establish possible biomarkers of HD in TgHD spermatozoa expressing the N-terminal part of mutated human Htt. A previous study in TgHD boars found decreased reproduction parameters [24] and a follow-up study confirmed this finding, showing an altered spermiogram and in vitro fertilization tests [20]. However, it was not clear whether mtHtt affects TgHD reproduction directly in germ cells and whether the pathologies arise from an altered MM of TgHD spermatozoa.

First of all, we detected that the polyQ signal of mtHtt (previously shown on Western blot [20]) is prominent in TgHD spermatozoa and localizes along the flagellum, including the mitochondria-rich midpiece (Fig. 1). This finding suggests that a direct interaction of mtHtt and mitochondria in spermatozoa is possible due to the proximity of both signals in the flagellum. Endogenous Htt was detectable on Western blot only by longer exposures and routinely displayed only a very weak signal in the area of molecular weight 320–340 kDa (Htt) compared to a high abundance of mtHtt (115–117 kDa) and fragmented mtHtt (60–70 kDa; Fig. 2), suggesting a low rescue effect of endogenous Htt, and therefore a higher vulnerability of TgHD spermatozoa to mtHtt impact.

MM measurements were optimized for the use in spermatozoa. Then, in order to assess MM in TgHD spermatozoa and to identify HD biomarkers, we analyzed MEGS in 10 distinct incubations. These incubations were also expressed as incubation per CS activity ratios, or further

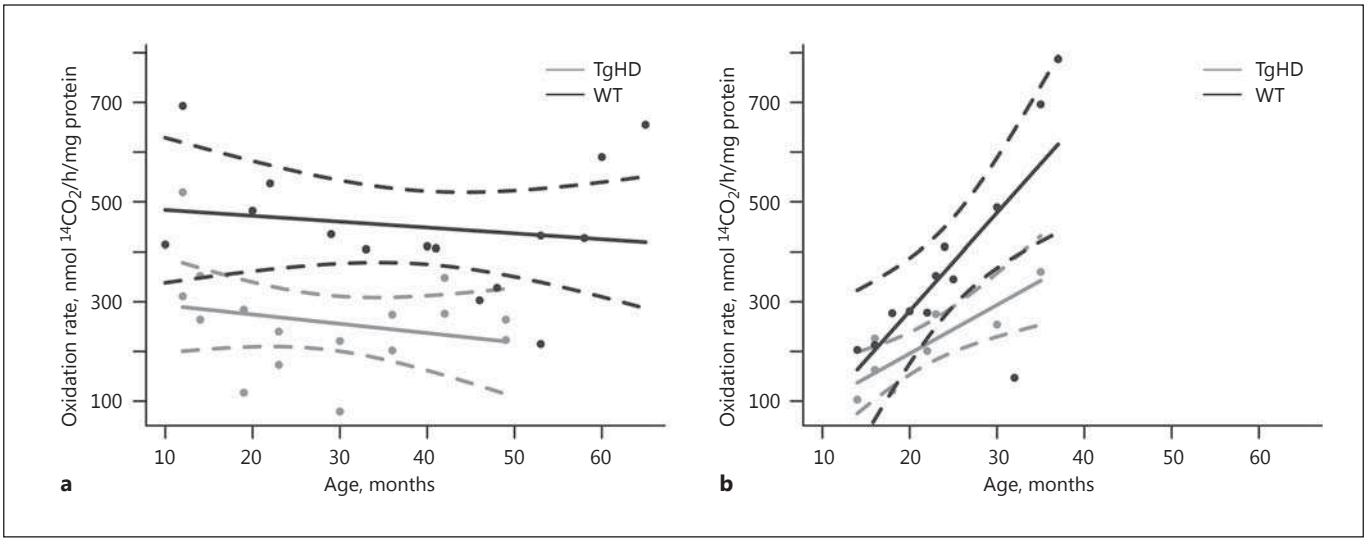


Fig. 3. Decreased mitochondrial energy-generating system capacity in TgHD boars in comparison to healthy controls (WT). In the MEGS incubation containing [1-^{14}C]pyruvate+carnitine+ADP (incubation 2), all HD-, age-, and generation-dependent differences were significant as shown; $p < 0.05$. **a** Incubation 2, MEGS activity dependent on [1-^{14}C]pyruvate+carnitine+ADP in F1 generation. **b** Incubation 2, MEGS activity dependent on [1-^{14}C]pyruvate+carnitine+ADP in F2 generation. In both generations, the activity was significantly lowered in TgHD boars' spermatozoa (light gray). However, the observed parameter depended not only on HD status, but was also age-related and showed a different progression between generations (notice the opposite slope of the linear regression). Analysis was performed in 12 boars ($n_{TgHD} = 6$, $n_{WT} = 6$) repeatedly, according to Table 1.

analyzed as ratios between certain incubations according to Janssen et al. [28]. MEGS is a method for complex functional analyses of mitochondrial energetics. In this method, every incubation provides a piece of information, but the set of 10 incubations, which combine radiolabeled substrate and other substrates, inhibitors, and uncouplers, provides an opportunity to check the TCA cycle, OXPHOS, and other enzymes' activity in general, and moreover to pinpoint the source of observed metabolic defect. For example, decreased oxidation of pyruvate (incubation 1) could be a result of a defect anywhere in the TCA cycle and respiratory chain, except complex II [28]. However, when acetyl-CoA is removed by carnitine acetyltransferase (as in incubation 2), the oxidation of pyruvate in OXPHOS deficiency will be less decreased than in incubation 1, so the ratio of these incubations (2/1) will be increased. However, in the case of PDHc deficiency, the 2/1 ratio remains approximately 1. The complete theory of this method is available in Janssen et al. [28]. Here we show, for the first time, measurements of MEGS in spermatozoa, slightly modifying and combining the methods frequently used in skeletal muscle and fibroblasts [28, 35, 36]. The MEGS capacity was measured in spermatozoal homogenates, unlike the postnuclear fraction routinely used in skeletal muscle tissue analyses. It was also measured without detergent agents, which are usually used in the case of fibroblast measurements. The cell protein content in MEGS homogenate, ranging between 20 and 80 μg in 1 reaction, was shown to be enough to get valuable substrate oxidation results. We also analyzed spermatozoal respiration, which was measured in 5 states corrected by background respiration and in specific respiratory ratios (Table 3). Analysis of respiration, just like MEGS measurements, can reveal which OXPHOS complex has a changed activity – we measured respiration caused by oxygen reduction on complex IV and combined substrates forming NADH, which is oxidized on complex I, or succinate forming $FADH_2$, which is oxidized on complex II. Because we know that OXPHOS is highly coupled, a defect in any of the complexes I–V will result in decreased respiration. Respiration was assessed in digitonin-permeabilized spermatozoa. The optimal digitonin concentration was established by manual titration according to a major respiration drop after plasma membrane rupture. These spermatozoa show less than 10% of the respiration rate of intact spermatozoa after the addition of approximately 4.1 μM of digitonin (1 μL stock solution diluted in DMSO). The addition of cytochrome *c* was used to check the intactness of mitochondrial membranes.

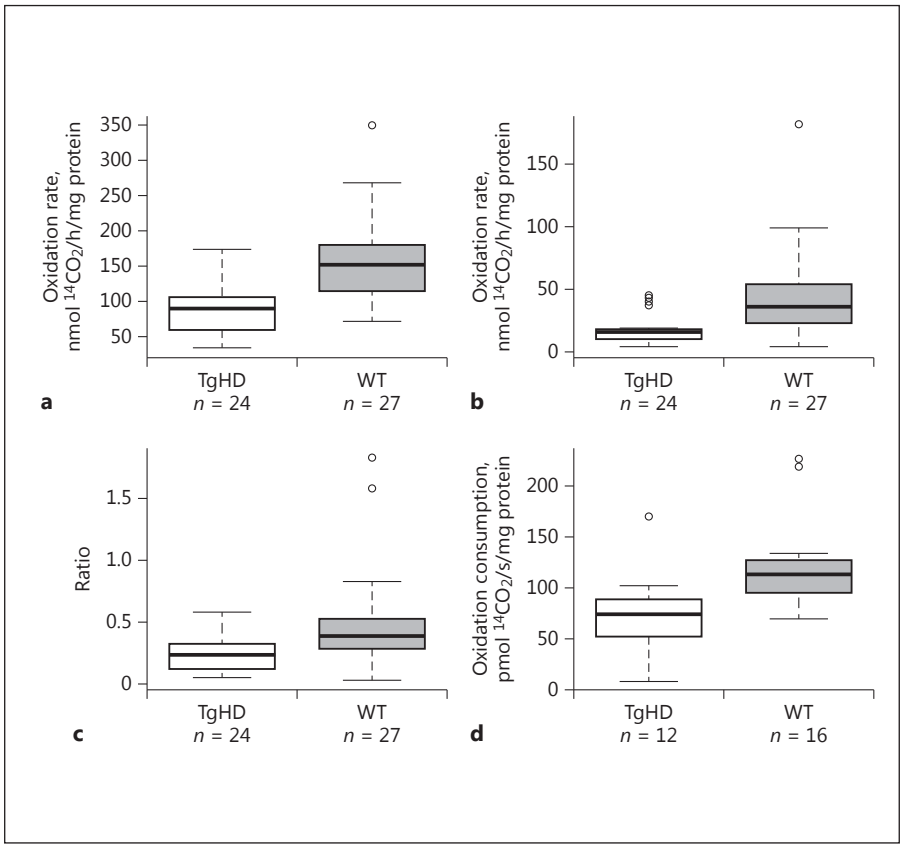


Fig. 4. Selected parameters of MEGS capacity and respiratory parameters, which showed significantly lowered values ($p < 0.05$) in TgHD boars' spermatozoa in comparison to healthy controls (WT). These parameters were not inherent to generation status and the age of the boar at the time of measurement. Incubations 4a and 6 – MEGS oxidation rates dependent on [1-^{14}C]pyruvate (incubation 4a; **a**) and [U-^{14}C]malate+pyruvate+malonate (incubation 6; **b**) in TgHD (white; $n_{TgHD} = 6$) compared to WT (gray; $n_{WT} = 6$) spermatozoa. **c** Incubation 6 expressed as a ratio to citrate synthase activity (6/CS). **d** Complex I-dependent respiration after glutamate+malate+ADP+cytochrome *c* corrected by background rotenone-insensitive residual respiration (GMDc-rot) in TgHD (white; $n_{TgHD} = 4$) compared to WT (gray; $n_{WT} = 4$) spermatozoa. In the sample set, the oxidation rates in incubations 4a and 6 were significantly lower in TgHD spermatozoa. TgHD spermatozoa also showed a diminished 6/CS ratio as well as complex I-dependent respiration. No correlation with age or generation was observed, which encourages using these parameters in further monitoring of HD in TgHD spermatozoa.

In order to analyze the experimental data, we concentrated on a statistical analysis of 3 possibly interfering factors: (a) the effect of HD itself, (b) the effect of generation, and (c) the effect of aging (Table 3). The statistical analysis revealed possible differences between TgHD and WT spermatozoa, and identified one or more effects influencing this observation.

Out of 36 parameters measured (Table 3), only 8 were related to a distinct generation (F1 or F2). These 8 parameters were also influenced by the "aging" effect. Five parameters (incubations 2, 4a, and 6; the 6/CS ratio; complex I-dependent respiration) showed a significant difference between the TgHD and WT samples. Incubation 2 ([1-^{14}C]pyruvate+carnitine+ADP) was decreased in TgHD animals but also significantly depended on the generation status and the age of the animal (Fig. 3), probably because it was not yet possible to analyze individuals of the F2 generation older than 36 months. Incubations 4a ([1-^{14}C]pyruvate+ADP; Fig. 4a), 6 ([U-^{14}C]malate+pyruvate+malonate+ADP; Fig. 4b), the 6/CS ratio (Fig. 4c) and complex I-dependent respiration after GMDc-rot (glutamate +malate+ADP+cytochrome *c*; Fig. 4d) were significantly diminished in TgHD animals. We discovered that 13 parameters might be associated with aging (Table 3): incubations 7 and 8, ratios 7/CS, 8/CS, and 6/1 were affected solely by the age of the animal and declined with age.

SDS-PAGE immunoblots of selected OXPHOS and PDHc subunits in spermatozoa obtained at the age of 47 months did not show major disturbances in PDHc subunit content. Only a slightly diminished content of the SDHA subunit (SDH70 of complex II) and aconitase 2 was detected in the spermatozoa of both TgHD individuals (Fig. 5a). In the case of SDHA, we observed that the amount of the subunit diminished with age in TgHD. Comparing the spermatozoa from the same boars at the age of 14 and 47 months, the amount reached about 50% of the age-related control (Fig. 5b, c). Although only the SDHA subunit amount was lower on Western blot, in-gel histochemistry assay after BN-PAGE revealed a slight disproportion in the activity of OXPHOS complexes among samples: already at the age of 14 months, the activities of complexes II and IV were lower, whereas the activities of complexes I and V were higher in TgHD in comparison with WT spermatozoa (Fig. 6).

All the methods used were optimized and proved to be suitable for the analysis of MM in minipig spermatozoa.

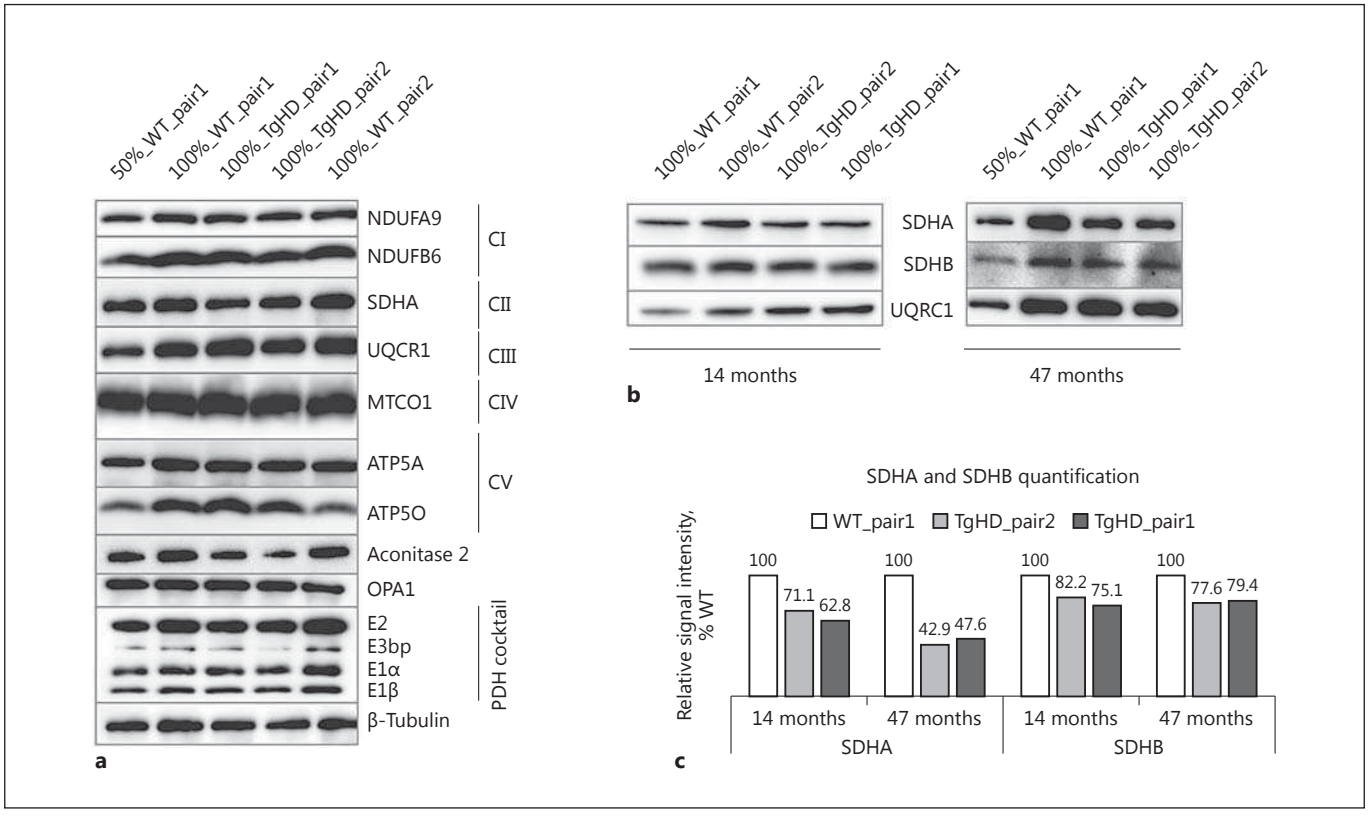


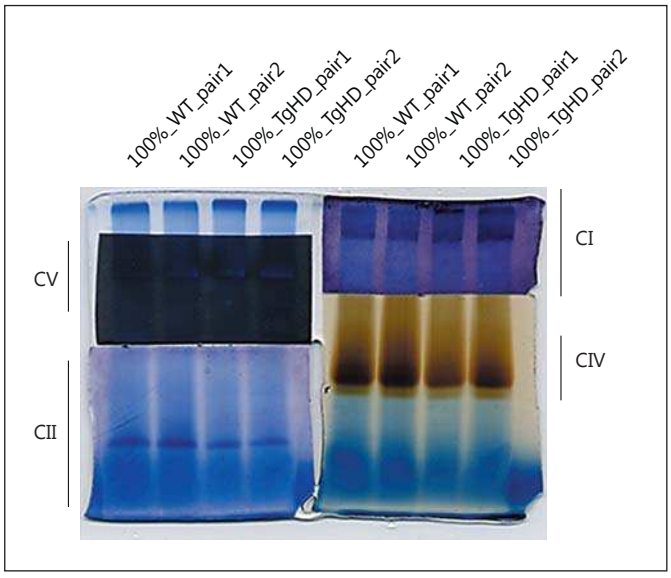
Fig. 5. Analysis of selected mitochondrial proteins revealed minor changes in the content of the OXPHOS system and PDHc subunits. **a** SDS-PAGE and immunodetection of OXPHOS and PDHc subunit content in TgHD boars' spermatozoa obtained at the age of 47 months did not show major disturbances in PDHc subunit content. Only slightly diminished content of SDHA subunit (SDH70; CII) and aconitase 2 were detected in TgHD spermatozoa. CI–V, OXPHOS complexes I–V; CI, NADH:ubiquinone oxidoreductase; CII, succinate dehydrogenase; CIII, cytochrome bc_1 complex; CIV, cytochrome *c* oxidase; CV, ATP synthase. **b**, **c** Age-dependent changes of complex II subunits SDHA and SDHB content in TgHD spermatozoa in comparison with WT: SDS-PAGE immunoblot showing a disturbance in SDHA and SDHB subunit content in spermatozoa at the age of 14 (left) and 47 (right) months; UQCR1 was used as the loading control. A slightly diminished content of SDHA and SDHB subunit was detected in TgHD spermatozoa. A time-course progression in the SDHA subunit content was observed. The linearity of the immunodetection signal was checked by loading both 5 μg (50%) and 10 μg (100%) of the whole protein in the sperm lysate, hence the samples are identified by the label: loading_WT/TgHD_pair number. $n_{TgHD} = 3$, $n_{WT} = 3$.

Although not all data clearly show a specific metabolic defect, the results identified 4 promising biomarkers of HD in TgHD spermatozoa.

Discussion

In this study, we analyzed the MM of transgenic minipig spermatozoa from boars carrying the N-terminal part of the human *HTT* gene on chromosome 1 [24]. Changes in MM might be an important biomarker of HD [37], useful in the phenotyping of our TgHD minipig model. We used methods that are widely applied in molecular diagnostics of mitochondrial disorders. This may help to elucidate the etiopathogenesis of HD in TgHD minipigs and to deepen our knowledge needed for further testing of new medications and HD therapy.

First, we documented that mtHtt is present in the spermatozoa and that it might be in direct interaction with their mitochondria (Fig. 1, 2). Our previous study showed that the germ epithelium of the tubuli seminiferi intensively expresses the WT Htt as well as mtHtt [20]. The anti-Htt EPR5526 antibody revealed the specific signal for mtHtt (115–117 kDa) [20]. However, only a very weak signal for endogenous Htt was obtained in both WT and TgHD spermatozoa (Fig. 2). Moreover, numerous small-



Color version available online

Fig. 6. Analysis of catalytic activity of OXPHOS complexes in TgHD boars' spermatozoa revealed only a slight differences in comparison to healthy controls (WT) at the age of 14 months. In-gel histochemistry assay analysis was performed using histochemical staining methods, following the separation of complexes by blue-native polyacrylamide gel electrophoresis. Slightly decreased activity of CII and CIV was observed. No major differences in other OXPHOS complex activities were detected; 60 μg (100 %) of solubilized protein per lane was resolved. The samples are identified by the label: loading_WT/TgHD_pair number. n_{TgHD} = 2, n_{WT} = 2. CI, NADH:ubiquinone oxidoreductase (deep blue stain); CII, succinate dehydrogenase (light blue stain); CIV, cytochrome *c* oxidase (brown stain); CV, ATP synthase (white opalescent stain on dark field).

er mtHtt fragments were documented in TgHD spermatozoa (60–70 kDa). These fragments were already detected in TgHD minipig cortex and testes, but were absent in skeletal muscle [38]. The presence of mtHtt fragments points to its possible gain of toxic function specifically in brain and testes tissues.

Our study involved a comparatively low number of animals when contrasted with studies on small-animal models (mice, *Drosophila*, etc.). In the subsequent analyses (MEGS, respiration), we therefore adhered to a special pairwise statistical analysis. For each measurement, an age-related control with the same or a highly similar genetic background to the TgHD subject was measured (e.g., siblings or very close relatives). Moreover, we performed an analysis of 3 possibly interfering factors (HD, generation, age).

Focusing on the effect of generation status, only 8 out of 36 parameters in the analysis were influenced by the generation to which the animals belonged. Thus, we predict that the phenotype is rather stable and it is not expected that major CAG expansions or deletions will occur in this TgHD minipig model upon transgene transmission between generations compared to the CAG triplet expansion instability detected in some TgHD mice [39–41]. This is in agreement with previous results suggesting that the mixed CAG/CAA triplet expansion in our TgHD minipig model is genetically stable [20; Eide and Klungland, unpubl. data].

To assess which parameters could be influenced by physiological MM decline observed during aging [42], the effect of aging was included in the statistical analysis. Those parameters which were significantly affected by the age of the animal at the time of measurement could be used only in comparison with proper age-related controls, which is an important detail for preclinical studies but hardly useful in clinical diagnostics of rare disorders in patients. We showed that incubations 7 and 8, and ratios 7/CS, 8/CS, and 6/1, all of which significantly declined with age in both WT and TgHD animals but were not linked with the HD genotype, can be considered as markers of the physiological decline in spermatozoal function during aging in our minipig model. A decline in fertility (the aging effect) was also reported in other mammals [43–46].

Finally, the effect of HD, demonstrated by a significant reduction of some parameters of MEGS capacity and respiration in TgHD boars, pointed to 5 parameters useful in HD progression monitoring and potential MM biomarkers of HD in the transgenic boars: MEGS incubations 2 ([1-^{14}C]pyruvate+carnitine+ADP; Fig. 3), 4a ([1-^{14}C]pyruvate+ADP; Fig. 4a), 6 ([U-^{14}C]malate+pyruvate+malonate+ADP; Fig. 4b), 6/CS ratio (Fig. 4c), and complex I-dependent respiration in permeabilized spermatozoa (Fig. 4d; see Table 3). Special attention must be paid in the case of incubation 2 (Fig. 3), which was decreased in TgHD spermatozoa, but significantly depended on (F1/F2) generation status and the age of the animal (Table 3). In TgHD boars, this parameter can therefore be monitored only in comparison with proper generation- and age-related control samples. Therefore, excluding incubation 2, incubations 4a, 6, 6/CS ratio, and complex I-dependent respiration significantly diminished in TgHD spermatozoa, suggesting that the observed defect of spermatozoal metabolism is rather complex and not related to a single mitochondrial metabolic pathway.

Incubations 4a, 6, and the 6/CS ratio, which all contained pyruvate, were diminished as well as complex I-dependent respiration, which leads to suspicion that the defect is linked to PDH – or complex I – deficiency. How-

ever, the decrease of ratio 6/1, which is typical in patients suffering from complex I deficiency (complex II is inhibited in incubation 6) [28], correlates only with age and is not affected by HD. On the other hand, increased sensitivity to the inhibition of complex I-dependent respiration was shown in the skeletal muscle of R6/2 mice [47]. Because the pyruvate-containing incubations 2 and 4a are both significantly decreased regardless of L-carnitine presence in the reaction, we can hypothesize that the metabolic defect is rather independent of the rate of fatty acid β-oxidation [48].

Interestingly, on Western blot, the only observed disturbances in OXPHOS and TCA content did not show any complex I defect either. On the other hand, the observed decrease in SDHA and aconitase 2 content is in agreement with a number of HD studies, e.g., the decrease in SDHA and SDHB subunits in striatal patients' neurons [49] and also with studies mimicking HD by 3-nitropropionate treatment (complex II inhibitor) [50–53]. These studies propose that SDHB is particularly vulnerable to oxidative stress in HD because of the presence of Fe-S clusters in its structure. A similar vulnerability to oxidative stress was shown even in aconitase (also containing Fe-S clusters), also by a decrease of aconitase expression in both humans and mice [54, 55]. Although we found only a mild decrease in SDH via histochemical staining (Fig. 6), we hypothesize that the observed decline of SDH and aconitase subunit content is an early response to oxidative stress, probably still compensated by an unknown mechanism during the presymptomatic stage of HD.

Considering the ongoing discussion about whether mammalian spermatozoa rely on OXPHOS or glycolysis [25, 26, 56–58], it is necessary to highlight the possible alternative fate of pyruvate in spermatozoa compared to somatic cells. Pyruvate, which is also available in the female reproductive tract [56], has been previously described as an exogenous source of NAD^+ [57]. NAD^+ is regenerated from NADH in cytosol of spermatozoa by a testes-specific enzyme lactate dehydrogenase (LDH-C), which increases the NAD^+/NADH ratio and accelerates glycolysis. Conversely, the mitochondrially located LDH-C can effectively oxidize lactate, which was produced in glycolysis and transported to the mitochondrial matrix, back to pyruvate, thus producing NADH and therefore directly providing 2 electrons to complex I. This was shown not only in skeletal muscle, but also in boar spermatozoa [59, 60]. LDH-C is localized in the principal piece of the sperm flagellum, but a weak signal was also detected in the nucleus and the midpiece [61, 62]. Its activity was found to be significantly decreased in low motile human spermatozoa [63]. Lactate-dependent gluconeogenesis in the liver was recently found to be distorted in R6/2 TgHD mice [64], but to our knowledge no analyses of LDH-C have so far been performed in TgHD spermatozoa.

To summarize, even though we could not pinpoint a simple metabolic defect responsible for the phenotype found in TgHD spermatozoa, we revealed that differences in sperm motility, progressivity, and in vitro penetration parameters observed in 13-month-old TgHD boars, which were unrelated to levels of fertility-related hormones or libido as presented previously [20], are also not caused solely by the overall ultrastructural deformities of TgHD spermatozoa as might be expected. Htt, which is also a component of a large variety of cilia and flagella, is involved in both nonmotile and motile cilia biogenesis that is altered in HD [65, 66]. Interestingly, the retina photoreceptor cilia in R6/2 mice show numerous structural and functional defects correlating with a strong reduction of endogenous Htt in cilia [66]. However, no pathologies were detected in sperm centriole in our TgHD minipigs [Sedlackova, unpubl. data]. Therefore, we suggest that observed spermatozoal pathologies are probably connected with the presence of abundantly expressed mtHtt (and its fragments) compared to endogenous Htt. This discrepancy, in our opinion, is conducive to the functional disturbances in the energy-producing metabolism of the cell, either MM and/or glycolysis [67], the decrease of which has also been shown in HD brains [68]. MEGS capacity measurements – incubations 4a, 6, 6/CS ratio, as well as complex I-dependent respiration of digitonin-permeabilized spermatozoa – are useful biomarkers of HD progression in our TgHD minipig model. Furthermore, these promising factors could be used in the monitoring of preclinical HD therapeutic treatment efficiency in a noninvasive, longitudinal approach.

Our complex biochemical approach to the study of TgHD minipig spermatozoa suggests the possibility that the small toxic fragments of mtHtt might interact with the cellular metabolism to significantly alter the MM of spermatozoa and that the mitochondrial dysfunction may cause the impaired penetration activity of spermatozoa, the first preclinical marker of HD in TgHD boars.

Conclusions

Our previous research showed that TgHD spermatozoa have a lower ability to penetrate zona pellucida, which leads to fertility decline. To elucidate whether this pathol-

ogy is connected with any metabolic defect, we decided to analyze the MM of TgHD spermatozoa in detail. During the longitudinal study, we identified 4 biomarkers which indicate a nonspecific pathology, perhaps in complex I of the OXPHOS system and/or in the glycolytic metabolism, possibly interconnected with the gain of toxic function of small mtHtt fragments that are present exclusively in the TgHD brain and testes. Spermatozoa are a noninvasively obtained material suitable for the monitoring of HD disease progression and harbor a great potential for efficient future therapy in the TgHD minipig model and beyond.

Acknowledgements

The authors are grateful to Dr. Vaclav Capek for advanced statistical analysis of the data and Dr. Robert Pacifici (CHDI Foundation) for his valuable comments on the manuscript. This study was supported by the Norwegian Financial Mechanism 2009–2014 and the Ministry of Education, Youth and Sports under Project Contract No. MSMT-28477/2014 "HUNTINGTON" 7F14308; NPU 1609 (MSMT); CHDI Foundation (A-8248, A-5378); PRVOUK P24/205024-4; GACR 14-36804G, Centre of Mitochondrial Biology and Pathology (MITOCENTRE), and by institutional projects UNCE 204011 and SVV UK 260256/2016. The authors of this paper are members of COST (European Cooperation in Science and Technology) Action BM1308 Sharing Advances on Large Animal Models (SALAAM; http://www.salaam.genzentrum.lmu.de).

Disclosure Statement

Dr. David S. Howland is employed by the CHDI Foundation. However, he was not involved in designing the experiments or making any decision to publish the work. He was our consultant. The authors have no other conflicts of interest to declare.

Author Contributions

M.M., B.B., and A.P. performed semen collection and spermiogram analysis; J.Kl. stained immunofluorescence signals; H.H. analyzed MEGS capacity; J.Kr. carried out respirometry; H.S., M.R., and D.V. performed Western blot immunodetection and histochemistry. Z.E., A.P., J.Z., J.M., and H.H. designed the experiments. All authors analyzed the data. J.Kr., Z.E., J.M., D.S.H., and H.H. wrote the manuscript.

References

1 MacDonald ME, Ambrose CM, Duyao MP, et al: A novel gene containing a trinucleotide repeat that is expanded and unstable on Huntington's disease chromosomes. Cell 1993;72:971–983.
2 Reiner A, Dragatsis I, Zeitlin S, Goldowitz D: Wild-type huntingtin plays a role in brain development and neuronal survival. Mol Neurobiol 2003;28:259–276.
3 Trushina E, Dyer RB, Badger JD, Ure D, Eide L, Tran DD, Vrieze BT, Legendre-Guillemin V, McPherson PS, Mandavilli BS, Van Houten B, Zeitlin S, McNiven M, Aebersold R, Hayden M, Parisi JE, Seeberg E, Dragatsis I, Doyle K, Bender A, Chacko C, McMurray CT: Mutant huntingtin impairs axonal trafficking in mammalian neurons in vivo and in vitro. Mol Cell Biol 2004;24:8195–8209.
4 Jeong SJ, Kim M, Chang KA, Kim HS, Park CH, Suh YH: Huntingtin is localized in the nucleus during preimplanatation embryo development in mice. Int J Dev Neurosci 2006;24:81–85.
5 Lumsden AL, Henshall TL, Dayan S, Lardelli MT, Richards RI: Huntingtin-deficient zebrafish exhibit defects in iron utilization and development. Hum Mol Genet 2007;16:1905–1920.
6 Yan JT, Zhang H, Liu Y, Zhao FL, Zhu S, Xie CM, Tang TS, Guo CX: Germline deletion of huntingtin causes male infertility and arrested spermiogenesis in mice. J Cell Sci 2016;129:492–501.
7 Walker FO: Huntington's disease. Lancet 2007;369:218–228.
8 Francelle L, Galvan L, Brouillet E: Possible involvement of self-defense mechanisms in the preferential vulnerability of the striatum in Huntington's disease. Front Cell Neurosci 2014;8:295.
9 Plotkin J, Surmeier D: Corticostriatal synaptic adaptations in Huntington's disease. Curr Opin Neurobiol 2015;33:53–62.
10 Sepers M, Raymond L: Mechanisms of synaptic dysfunction and excitotoxicity in Huntington's disease. Drug Discov Today 2014;19:990–996.
11 Hinckelmann M, Zala D, Saudou F: Releasing the brake: restoring fast axonal transport in neurodegenerative disorders. Trends Cell Biol 2013;23:634–643.
12 Costa V, Scorrano L: Shaping the role of mitochondria in the pathogenesis of Huntington's disease. EMBO J 2012;31:1853–1864.
13 Oliveira JM: Mitochondrial bioenergetics and dynamics in Huntington's disease: tripartite synapses and selective striatal degeneration. J Bioenerg Biomembr 2010;42:227–234.
14 Reddy PH, Shirendeb UP: Mutant huntingtin, abnormal mitochondrial dynamics, defective axonal transport of mitochondria, and selective synaptic degeneration in Huntington's disease. Biochim Biophys Acta 2012;1822:101–110.
15 Bolaños JP, Heales SJ: Persistent mitochondrial damage by nitric oxide and its derivatives: neuropathological implications. Front Neuroenergetics 2010;2:1.
16 Oliveira J: Techniques to investigate neuronal mitochondrial function and its pharmacological modulation. Curr Drug Targets 2011;12:762–773.
17 Kim J, Moody JP, Edgerly CK, Bordiuk OL, Cormier K, Smith K, Beal MF, Ferrante RJ: Mitochondrial loss, dysfunction and altered dynamics in Huntington's disease. Hum Mol Genet 2010;19:3919–3935.
18 Im W, Chung J, Lee ST, Chu K, Kim MW, Kim M: Nuclear localization of huntingtin during spermatogenesis. Neurol Sci 2014;35:459–462.
19 Guo J, Zhu P, Wu C, Yu L, Zhao S, Gu X: In silico analysis indicates a similar gene expression pattern between human brain and testis. Cytogenet Genome Res 2003;103:58–62.
20 Macakova M, Bohuslavova B, Vochozkova P, Pavlok A, Sedlackova M, Vidinska D, Vochyanova K, Liskova I, Valekova I, Baxa M, Ellederova Z, Klima J, Juhas S, Juhasova J, Klouckova J, Haluzik M, Klempir J, Hansikova H, Spacilova J, Collins R, Blumenthal I, Talkowski M, Gusella JF, Howland DS, DiFiglia M, Motlik J: Mutated huntingtin causes testicular pathology in transgenic minipig boars. Neurodegener Dis 2016;16:245–259.

21 van Raamsdonk JM, Murphy Z, Selva DM, Hamidizadeh R, Pearson J, Petersen A, Bjorkqvist M, Muir C, Mackenzie IR, Hammond GL, Vogl AW, Hayden MR, Leavitt BR: Testicular degeneration in Huntington disease. Neurobiol Dis 2007;26:512–520.
22 van Raamsdonk JM, Metzler M, Slow E, Pearson J, Schwab C, Carroll J, Graham RK, Leavitt BR, Hayden MR: Phenotypic abnormalities in the YAC128 mouse model of Huntington disease are penetrant on multiple genetic backgrounds and modulated by strain. Neurobiol Dis 2007;26:189–200.
23 Macakova M, Hansikova H, Antonin P, Hajkova Z, Sadkova J, Juha S, Juhasova J, Baxa M, Zeman J, Motlik J: Reproductive parameters and mitochondrial function in spermatozoa of F1 and F2 minipig boars transgenic for n-terminal part of the human mutated huntingtin. J Neurol Neurosurg Psychiatry 2012; 83:A16.
24 Baxa M, Hruska-Plochan M, Juhas S, Vodicka P, Pavlok A, Juhasova J, Miyanohara A, Nejime T, Klima J, Macakova M, Marsala S, Weiss A, Kubickova S, Musilova P, Vrtel R, Sontag EM, Thompson LM, Schier J, Hansikova H, Howland DS, Cattaneo E, DiFiglia M, Marsala M, Motlik J: A transgenic minipig model of Huntington's disease. J Huntingtons Dis 2013;2:47–68.
25 Mukai C, Okuno M: Glycolysis plays a major role for adenosine triphosphate supplementation in mouse sperm flagellar movement. Biol Reprod 2004;71:540–547.
26 Stendardi A, Focarelli R, Piomboni P, Palumberi D, Serafini F, Ferramosca A, Zara V: Evaluation of mitochondrial respiratory efficiency during in vitro capacitation of human spermatozoa. Int J Androl 2011;34:247–255.
27 Schmidt H, Kamp G: Induced hyperactivity in boar spermatozoa and its evaluation by computer-assisted sperm analysis. Reproduction 2004;128:171–179.
28 Janssen AJ, Trijbels FJ, Sengers RC, Wintjes LT, Ruitenbeek W, Smeitink JA, Morava E, van Engelen BG, van den Heuvel LP, Rodenburg RJ: Measurement of the energy-generating capacity of human muscle mitochondria: diagnostic procedure and application to human pathology. Clin Chem 2006;52:860–871.
29 Lowry OH, Rosebrough NJ, Farr AL, Randall RJ: Protein measurement with the folin phenol reagent. J Biol Chem 1951;193:265–275.
30 Kuznetsov AV, Veksler V, Gellerich FN, Saks V, Margreiter R, Kunz WS: Analysis of mitochondrial function in situ in permeabilized muscle fibers, tissues and cells. Nat Protoc 2008;3:965–976.
31 Stiburek L, Fornuskova D, Wenchich L, Pejznochova M, Hansikova H, Zeman J: Knockdown of human Oxa1l impairs the biogenesis of F_1F_o-ATP synthase and NADH:ubiquinone oxidoreductase. J Mol Biol 2007;374:506–516.
32 Rustin P, Chretien D, Bourgeron T, Gérard B, Rötig A, Saudubray JM, Munnich A: Biochemical and molecular investigations in respiratory chain deficiencies. Clin Chim Acta 1994;228:35–51.
33 Schägger H, von Jagow G: Blue native electrophoresis for isolation of membrane protein complexes in enzymatically active form. Anal Biochem 1991;199:223–231.
34 van Coster R, Smet J, George E, De Meirleir L, Seneca S, van Hove J, Sebire G, Verhelst H, de Bleecker J, van Vlem B, Verloo P, Leroy J: Blue native polyacrylamide gel electrophoresis: a powerful tool in diagnosis of oxidative phosphorylation defects. Pediatr Res 2001;50:658–665.
35 Janssen AJ, Schuelke M, Smeitink JA, Trijbels FJ, Sengers RC, Lucke B, Wintjes LT, Morava E, van Engelen BG, Smits BW, Hol FA, Siers MH, Ter Laak H, van der Knaap MS, van Spronsen FJ, Rodenburg RJ, van den Heuvel LP: Muscle 3243A→G mutation load and capacity of the mitochondrial energy-generating system. Ann Neurol 2008;63:473–481.
36 Cameron JM, Levandovskiy V, MacKay N, Robinson BH: Respiratory chain analysis of skin fibroblasts in mitochondrial disease. Mitochondrion 2004;4:387–394.
37 Oliveira JM, Jekabsons MB, Chen S, Lin A, Rego AC, Goncalves J, Ellerby LM, Nicholls DG: Mitochondrial dysfunction in Huntington's disease: the bioenergetics of isolated and in situ mitochondria from transgenic mice. J Neurochem 2007;101:241–249.
38 Vidinska D, Motlik J, Ellederova Z: Different forms of huntingtin in the most affected organs; brain and testes of transgenic minipigs. Ceska Slov Neurol N 2015;78:2S66–2S69.
39 Møllersen L, Rowe AD, Larsen E, Rognes T, Klungland A: Continuous and periodic expansion of cag repeats in Huntington's disease R6/1 mice. PLoS Genet 2010;6:e1001242.
40 Jonson I, Ougland R, Klungland A, Larsen E: Oxidative stress causes DNA triplet expansion in Huntington's disease mouse embryonic stem cells. Stem Cell Res 2013;11:1264–1271.
41 Mangiarini L, Sathasivam K, Seller M, Cozens B, Harper A, Hetherington C, Lawton M, Trottier Y, Lehrach H, Davies SW, Bates GP: Exon 1 of the *HD* gene with an expanded cag repeat is sufficient to cause a progressive neurological phenotype in transgenic mice. Cell 1996;87:493–506.
42 Nicholls DG: Mitochondrial function and dysfunction in the cell: its relevance to aging and aging-related disease. Int J Biochem Cell Biol 2002;34:1372–1381.
43 Ozkosem B, Feinstein SI, Fisher AB, O'Flaherty C: Advancing age increases sperm chromatin damage and impairs fertility in peroxiredoxin 6 null mice. Redox Biol 2015;5: 15–23.
44 Syntin P, Robaire B: Sperm structural and motility changes during aging in the brown Norway rat. J Androl 2001;22:235–244.
45 Jung A, Schuppe HC, Schill WB: Comparison of semen quality in older and younger men attending an andrology clinic. Andrologia 2002;34:116–122.
46 Weir CP, Robaire B: Spermatozoa have decreased antioxidant enzymatic capacity and increased reactive oxygen species production during aging in the brown Norway rat. J Androl 2007;28:229–240.
47 Gizatullina ZZ, Lindenberg KS, Harjes P, Chen Y, Kosinski CM, Landwehrmeyer BG, Ludolph AC, Striggow F, Zierz S, Gellerich FN: Low stability of Huntington muscle mitochondria against Ca^{2+} in R6/2 mice. Ann Neurol 2006;59:407–411.
48 Brass EP, Hoppel CL: Relationship between acid-soluble carnitine and coenzyme A pools in vivo. Biochem J 1980;190:495–504.
49 Benchoua A, Trioulier Y, Zala D, Gaillard MC, Lefort N, Dufour N, Saudou F, Elalouf JM, Hirsch E, Hantraye P, Déglon N, Brouillet E: Involvement of mitochondrial complex II defects in neuronal death produced by N-terminus fragment of mutated huntingtin. Mol Biol Cell 2006;17:1652–1663.
50 Túnez I, Tasset I, Pérez-De La Cruz V, Santamaría A: 3-Nitropropionic acid as a tool to study the mechanisms involved in Huntington's disease: past, present and future. Molecules 2010;15:878–916.
51 Ramaswamy S, McBride JL, Kordower JH: Animal models of Huntington's disease. ILAR J 2007;48:356–373.
52 Brouillet E, Guyot MC, Mittoux V, Altairac S, Condé F, Palfi S, Hantraye P: Partial inhibition of brain succinate dehydrogenase by 3-nitropropionic acid is sufficient to initiate striatal degeneration in rat. J Neurochem 1998;70:794–805.
53 Hariharan A, Shetty S, Shirole T, Jagtap AG: Potential of protease inhibitor in 3-nitropropionic acid induced Huntington's disease like symptoms: mitochondrial dysfunction and neurodegeneration. Neurotoxicology 2014; 45:139–148.
54 Sorolla MA, Reverter-Branchat G, Tamarit J, Ferrer I, Ros J, Cabiscol E: Proteomic and oxidative stress analysis in human brain samples of Huntington disease. Free Radic Biol Med 2008;45:667–678.
55 Perluigi M, Poon HF, Maragos W, Pierce WM, Klein JB, Calabrese V, Cini C, de Marco C, Butterfield DA: Proteomic analysis of protein expression and oxidative modification in R6/2 transgenic mice: a model of Huntington disease. Mol Cell Proteomics 2005;4:1849–1861.
56 Ruiz-Pesini E, Díez-Sánchez C, López-Pérez MJ, Enríquez JA: The role of the mitochondrion in sperm function: is there a place for oxidative phosphorylation or is this a purely glycolytic process? Curr Top Dev Biol 2007; 77:3–19.

57 Hereng TH, Elgstoen KBP, Cederkvist FH, Eide L, Jahnsen T, Skalhegg BS, Rosendal KR: Exogenous pyruvate accelerates glycolysis and promotes capacitation in human spermatozoa. Hum Reprod 2011;26:3249–3263.
58 Piomboni P, Focarelli R, Stendardi A, Ferramosca A, Zara V: The role of mitochondria in energy production for human sperm motility. Int J Androl 2012;35:109–124.
59 Brooks GA: Mammalian fuel utilization during sustained exercise. Comp Biochem Physiol B Biochem Mol Biol 1998;120:89–107.
60 Calvin J, Tubbs PK: Mitochondrial transport processes and oxidation of NADH by hypotonically-treated boar spermatozoa. Eur J Biochem 1978;89:315–320.
61 Goldberg E, Eddy EM, Duan C, Odet F: LDHC: the ultimate testis-specific gene. J Androl 2010;31:86–94.
62 Medrano A, Fernández-Novell JM, Ramió L, Alvarez J, Goldberg E, Montserrat Rivera M, Guinovart JJ, Rigau T, Rodríguez-Gil JE: Utilization of citrate and lactate through a lactate dehydrogenase and ATP-regulated pathway in boar spermatozoa. Mol Reprod Dev 2006; 73:369–378.
63 Wang H, Zhou Z, Xu M, Li J, Xiao J, Xu ZY, Sha J: A spermatogenesis-related gene expression profile in human spermatozoa and its potential clinical applications. J Mol Med 2004; 82:317–324.
64 Nielsen SM, Hasholt L, Nørremølle A, Josefsen K: Progressive impairment of lactate-based gluconeogenesis in the Huntington's disease mouse model R6/2. PLoS Curr 2015; 1:7.
65 Keryer G, Pineda JR, Liot G, Kim J, Dietrich P, Benstaali C, Smith K, Cordelières FP, Spassky N, Ferrante RJ, Dragatsis I, Saudou F: Ciliogenesis is regulated by a huntingtin-HAP1-PCM1 pathway and is altered in Huntington disease. J Clin Invest 2011;121:4372–4382.
66 Karam A, Tebbe L, Weber C, Messaddeq N, Morlé L, Kessler P, Wolfrum U, Trottier Y: A novel function of huntingtin in the cilium and retinal ciliopathy in Huntington's disease mice. Neurobiol Dis 2015;80:15–28.
67 Ismailoglu I, Chen Q, Popowski M, Yang L, Gross SS, Brivanlou AH: Huntingtin protein is essential for mitochondrial metabolism, bioenergetics and structure in murine embryonic stem cells. Dev Biol 2014;391:230–240.
68 Powers WJ, Videen TO, Markham J, McGee-Minnich L, Antenor-Dorsey JV, Hershey T, Perlmutter JS: Selective defect of in vivo glycolysis in early Huntington's disease striatum. Proc Natl Acad Sci USA 2007;104:2945–2949.