Univerza *v Ljubljani*
Veterinarska fakulteta

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TOKSIKOKINETIKA BISFENOLA A PRI OVCAH IN NJEGOV VPLIV NA SPOLNE ORGANE OVNOV V PUBERTETI

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TOXICOKINETICS OF BISPHENOL A IN SHEEP AND ITS EFFECTS ON REPRODUCTIVE ORGANS OF RAMS IN PUBERTY

Doctoral dissertation

Ljubljana, 2022

Sabina Šturm

Toksikokinetika bisfenola A pri ovcah in njegov vpliv na spolne organe ovnov v puberteti

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IZVLEČEK

Namen prvega sklopa doktorske naloge je bil preučiti toksikokinetične (TK) parametre pri ovci in ovnih pasme istrska pramenka po zaporednem dietarnem in podkožnem vnosu bisfenola A (BPA). Za določanje koncentracije prostega in celokupnega BPA v krvni plazmi, mleku, urinu in iztrebkih ovce ter za določanje koncentracije celokupnega BPA v krvni plazmi ovnov smo razvili in validirali tekočinsko kromatografijo visoke ločljivosti na obrnjeni stacionarni fazi ter s fluorescenčno detekcijo. V prvi, pilotni raziskavi, opravljeni na eni ovci v laktaciji s sesnim jagnjetom, smo na podlagi meritev koncentracije prostega in celokupnega BPA v krvni plazmi z neprostorsko TK analizo izračunali osnovne TK parametre po dietarnem in podkožnem vnosu v odmerku 100 µg/kg t. m./dan in ugotovili, da je način vnosa vplival na TK parametre prostega BPA pri ovci. Po dietarnem vnosu je bila izpostavljenost ovce prostemu BPA manjša in krajša – površina pod koncentracijsko krivuljo v krvni plazmi (AUC) je bila 1,28 µg h/l v primerjavi s podkožnim vnosom, pri katerem je bila AUC prostega BPA 33,3 µg h/l. TK profila celokupnega BPA pa sta si bila zelo podobna. Z razvojem prostorskega TK modela smo ocenili, da se je v mleko izločilo manj kot 0,1 % odmerka, ki ga je prejela ovca, ne glede na način vnosa. V urinu ovce smo izmerili zelo nizke vrednosti prostega BPA in visoke vrednosti celokupnega BPA. V iztrebkih ovce smo izmerili zelo nizke vrednosti prostega in celokupnega BPA. Po štirikrat manjšem odmerku, ki so ga prejeli ovni, smo v krvni plazmi določili tudi približno štirikrat manjšo najvišjo koncentracijo (C_{max}) celokupnega BPA.

Namen drugega sklopa doktorske naloge je bil preučiti vpliv dvomesečne dietarne izpostavljenosti v odmerku BPA 25 µg/kg t. m./dan na spolne organe ovnov v puberteti. Med tretirano skupino in kontrolno skupino ovnov nismo ugotovili statistično značilnih razlik v telesni masi, masi mod, pogostosti ali jakosti histopatoloških sprememb v modih in nadmodkih, v kvalitativnih lastnostih semenčic ter koncentraciji semenčic v nadmodkih in semenovodih. Prav tako nismo ugotovili statistično značilnih razlik v premeru in površini zvitih semenskih kanalčkov. Statistično značilno razliko smo ugotovili le pri višini zarodnega epitela zvitih semenskih cevk, ki je bila nižja pri tretiranih ovnih.

Ključne besede: fenoli – analize; toksikokinetika; kromatografija, tekočinska; kemijske analize krvi; mleko – analize; urin – analize; feces – analize; reprodukcija; modo; nadmodek; ovce

ABSTRACT

The aim of the first part of the doctoral dissertation was to study toxicokinetic (TK) parameters in a ewe and rams of the Istrian pramenka sheep breed after sequential dietary and/or subcutaneous administration of bisphenol A (BPA). A chemical analysis using high performance liquid chromatography with reversed stationary phase and fluorescence detection was developed and validated for the determination of free and total BPA in sheep's blood plasma, milk, urine and feces. In an initial pilot study conducted on a lactating ewe with a suckling lamb, a non-compartmental TK analysis was performed for both the dietary and the subcutaneous route. In each case, 100 µg BPA/kg body weight/day was administered, and the free and total BPA in the blood plasma was determined. It was found that the administration route affected the TK parameters of free BPA. After dietary administration, the ewe's exposure to free BPA was lower and shorter - the area under the blood plasma concentration curve (AUC) was 1.28 µg h/l, compared to subcutaneous administration, where the AUC of free BPA was 33.3 µg h/l. However, the TK profiles of total BPA were very similar. Moreover, with the development of the TK compartmental model, we estimated that the percentage of BPA excreted in milk was less than 0.1% of the received dose, regardless of the route of administration. In the urine of the ewe we also determined very low levels of free BPA and high levels of total BPA, while very low levels of both free and total BPA were also determined in the feces. After a fourfold reduction of the dose, which was administered to the rams, an approximately fourfold reduction of the maximum plasma concentration (C_{max}) of total BPA in their blood plasma was also observed.

The second part of the dissertation aimed to investigate the effect of a two-month dietary exposure of BPA to 25 µg/kg of body weight/day on the male reproductive organs of pubescent rams. We found no statistically significant difference between the treatment and control group of rams regarding body weight, testicular weight, frequency or severity of histopathological changes in the testes and epididymis, qualitative characteristics of spermatozoa, concentration of spermatozoa in the epididymis and in the ductus deferens. We also found no statistically significant difference in the diameter and surface area of the seminiferous tubules. The only statistically significant difference was a lower height of the germinal epithelium of the seminiferous tubules in the treated rams compared to the control rams.

Key words: phenols – analysis; toxicokinetics; chromatography, high pressure liquid; blood chemical analysis; milk – analysis; urine – analysis; feces – analysis; reproduction; testis; epididymis; sheep

KAZALO VSEBINE

KAZALO SLIK

KAZALO OKRAJŠAV IN SIMBOLOV

^{14}C-BPA	bisfenol A, označen z radioaktivnim izotopom ogljika z masnim številom 14; *carbon-14 labeled bisphenol A*
AhR	aril ogljikovodikov receptor; *aryl hydrocarbon receptor*
AIC	informacijski kriterij Akaike; *Akaike information criterion*
CLARITY-BPA	konzorcij, ki povezuje akademska in regulativna spoznanja o toksičnosti BPA; *Consortium Linking Academic and Regulatory Insights on BPA Toxicity*
AUC	površina pod koncentracijsko krivuljo; *area under curve*
BPA	bisfenol A; *bisphenol A*
BPAG	bisfenol A glukuronid; *bisphenol A glucuronide*
BPF	bisfenol F; *bisphenol F*
BPS	bisfenol S; *bisphenol S*
CASA	računalniško podprt sistem za analizo semenčic; c*omputer assisted sperm analysis*
CDC	center za nadzor bolezni; *Center for Disease Control and Prevention*
C_{max}	najvišja ugotovljena plazemska koncentracija učinkovine; *maximum determined plasma concentration of compound*
EC	Evropska Komisija; *European Commission*
ECHA	Evropska agencija za kemikalije; *European Chemical Agency*
EFSA	Evropska agencija za varnost hrane; *European Food Safety Agency*
ELISA	encimski imunski test; *enzyme-linked immunosorbent assay*
EPA	Ameriška agencija za varstvo okolja; *Environmental Protection Agency*

ER	estrogenski receptor
Erα	estrogenski receptorji tipa alfa
ERβ	estrogenski receptorji tipa beta
ERRγ	estrogenskemu receptorju podoben receptor gama; *estrogen-related receptor γ*
FDA	Ameriška Uprava za hrano in zdravila; *Food and Drug Administration*
GC-MS/MS	plinska kromatografija s tandemsko masno spektrometrično detekcijo; *gas chromatography with tandem mass spectrometric detection*
HPLC	tekočinska kromatografija visoke ločljivosti; *high-performance liquid chromatography*
HPLC-FL	tekočinska kromatografija visoke ločljivosti s fluorescenčno detekcijo; *high-performance liquid chromatography with fluorescence detection*
HPLC-MS/MS	tekočinska kromatografija visoke ločljivosti s tandemsko masno spektrometrično detekcijo; *high performance liquid chromatography with tandem mass spectrometric detection*
KPHM	kemični povzročitelji hormonskih motenj
LLE	ekstrakcija tekoče-tekoče; *liquid-liquid extraction*
LOD	meja detekcije; *limit of detection*
LOQ	meja kvantifikacije; *limit of quantification*
MAE	ekstrakcija s pomočjo mikrovalov; *microwave-assisted extraction*
MIP	molekularno vtisnjeni polimeri; *molecularly imprinted polymers*

MISPE	ekstrakcija na trdni fazi z molekularnim odtisom; *molecularly imprinted solid phase extraction*
MSPD	disperzija trdne faze v matriksu; *matrix solid-phase dispersion*
NF - κB	jedrni dejavnik kapa-B; *nuclear factor kappa-B*
NOAEL	najvišji testni odmerek brez opaznega škodljivega učinka; *no-observed-adverse-effect level*
NOEC	najvišja koncentracija brez opaznega učinka; *no-observed-effect concentration*
PLE	tekočinska ekstrakcija pod visokim pritiskom; *pressurized liquid extraction*
PRX	pregnanski X receptor; *pregnane X receptor*
ROS	reaktivne kisikove zvrsti; *reactive oxygen species*
SBSE	sorptivna ekstrakcija z mešalnimi palicami; s*tir bar sorptive extraction*
SPE	ekstrakcija na trdni fazi; s*olid phase extraction*
SPME	mikroekstrakcija na trdnem nosilcu; s*olid phase microextraction*
TDI	sprejemljiv dnevni vnos; *tolerable daily intake*
TK	toksikokinetika
t_{max}	čas, ko je bila dosežena najvišja ugotovljena plazemska koncentracija učinkovine (C_{max}); *time to peak compound concentration (C_{max})*
TR	receptorji za ščitnične hormone; *thyroid hormone receptor*
t-TDI	začasno določen sprejemljiv dnevni vnos; *temporary tolerable daily intake*

1 UVOD

BPA je sestavni del polikarbonatne plastike in epoksi premazov. Nahaja se v embalaži za hrano in pijačo, zaviralcih gorenja, lepilih, gradbenem materialu, elektronskih komponentah, papirju za račune in v številnih drugih proizvodih (1). Slaba lastnost BPA je njegova zmožnost vplivanja na hormonski sistem živali in ljudi, zaradi česar ga uvrščamo med kemične povzročitelje hormonskih motenj (KPHM). KPHM so vnesene snovi oziroma zmesi snovi, ki s spremembami v delovanju hormonskega sistema povzročijo škodljive učinke na zdravje v intaktnega organizma, njegovega potomstva oziroma (sub)populacije. Škodljivi vplivi BPA pri visokih odmerkih veljajo za nedvomno dokazane, medtem ko predstavljajo vplivi nizkih koncentracij, ki smo jim vsakodnevno izpostavljeni, kontroverzno področje (2). Zaradi uporabnosti BPA in množične svetovne proizvodnje plastike in epoksi premazov ugotavljamo BPA, ki izhaja iz plastične embalaže, v hrani in pijači ter v različnih okoljskih vzorcih. Izločanje BPA v okolje je nevarno za ves ekosistem (3), potencialno tudi za živali za proizvodnjo živil, ki bi lahko v stik z BPA prišle prek kontaminirane vode, krme in okolja (4). Študije absorpcije, distribucije in izločanja BPA ter njegovega vpliva na zdravje ljudi so bile pretežno opravljene na laboratorijskih glodavcih, pri katerih so raziskovalci pogosto uporabljali nerealno visoke odmerke, parenteralni vnos ali vnos s sondo. Za opisane pristope nekateri raziskovalci domnevajo, da ne predstavljajo realnega vnosa BPA v organizem (5). Raziskovalce je zanimal tudi vpliv KPHM na zdravje živali, namenjenih za proizvodnjo živil. Ugotovili so, da nekateri KPHM lahko vplivajo na zdravstveno stanje takih živali. Uporaba alternativnih živalskih modelov pri preučevanju škodljivih učinkov KPHM je pomembna tudi zaradi ugotavljanja edinstvenosti škodljivih učinkov pri laboratorijskih živalih (6). Zanimivo je namreč, da se rezultati raziskav o vplivu BPA na različne organske sisteme močno razlikujejo že znotraj ene živalske vrste, npr. pri podganah različnih sojev (7). Eno izmed občutljivejših področij vplivanja BPA na zdravje predstavlja potencialen vpliv BPA na reproduktivno zdravje moških. Do sedaj so v nekaterih *in vivo* raziskavah, ki so preučevale toksičnost BPA na moške spolne organe in so bile večinoma opravljene na glodavcih, ugotovili vpliv BPA na spermatogenezo in seme živali (8), v drugih raziskavah pa tovrstnega škodljivega vpliva niso ugotovili (9).

Naše raziskave smo izvedli na domačih ovcah (*Ovis aries*) avtohtone slovenske pasme istrska pramenka. V prvem sklopu doktorske naloge smo preučevali TK BPA pri ovci v laktaciji z jagnjetom in ovnih. Oba postopka, postopek z ovco v laktaciji z jagnjetom in postopek z ovni, sta bila opravljena v Centru za sonaravno rekultiviranje Vremščica Univerze v Ljubljani

Veterinarske fakultete. Postopek na ovci je bil izveden za določitev načina vnosa in odmerka v postopku z ovni. Poleg tega smo v postopku z ovco preučevali tudi izločanje BPA z urinom, iztrebki in mlekom. Ker je mleko pomembno živilo za ljudi, v literaturi pa nismo zasledili raziskav, ki bi že dokazale izločanje BPA z mlekom pri prežvekovalcih, smo postavili našo prvo hipotezo, da je BPA v vzorcih ovce mogoče zaznati po dietarnem in podkožnem vnosu. V postopku z ovni pa smo želeli dokazati notranjo izpostavljenost ovnov BPA, zato smo prvi dan postopka pridobili zaporedne vzorce krvne plazme ovnov. Postavili smo drugo hipotezo, da način vnosa in fiziološko obdobje živali vplivata na TK profil BPA. Predpogoj za preučevanje TK pa je bil razvoj kemijskih analiznih metod za določanje koncentracije prostega BPA in celokupnega BPA v kompleksnih bioloških vzorcih. Izraz prosti BPA se v doktorski nalogi in v objavljenih znanstvenih delih nanaša na aglikon oz. nekonjugirani BPA, medtem ko se izraz celokupni BPA nanaša na vsoto aglikona in konjugiranega BPA, katerega glavni del je metabolit, BPA glukuronid (BPAG). Izraz celokupni BPA je bil izbran zaradi načina priprave vzorcev, tj. z encimsko dekonjugacijo glukuronidne vezi z encimom ß-glukuronidaza iz *Helix pomatia* vrste HP-2, ki smo ga uporabili v nalogi in s katerim najpravilneje navedemo rezultate analize.

V drugem sklopu doktorske naloge smo preučevali učinkovanje BPA na spolne organe ovnov v puberteti. Za ugotavljanje potencialnih škodljivih učinkov BPA smo opravili histopatološko preiskavo mod in nadmodkov, morfometrične meritve zvitih semenskih cevk in osnovne reprodukcijske teste semena. Poleg tega smo ugotavljali vpliv na telesno maso in maso mod ovnov. Vzorce za drugi sklop doktorske naloge smo pridobili iz postopka z ovni, ki jim je bila prvi dan odvzeta krvna plazma. Ovni so bili po dvomesečnem vsakodnevnem dietarnem vnosu BPA žrtvovani in raztelešeni. Med raztelesbo smo jim odvzeli moda, nadmodke in pripadajoče semenovode za prej navedene preiskave in teste. Glede na ugotovitve številnih raziskav, opravljenih na glodavcih, smo pričakovali, da bomo z izbranimi metodami dokazali škodljiv učinek BPA na spolne organe ovnov, zato je bila naša tretja hipoteza, da 64-dnevna izpostavljenost BPA s hrano v odmerku 25 µg/kg t. m. vpliva na morfološke značilnosti mod in osnovne parametre semena.

1.1 PREGLED LITERATURE

V vseh večceličnih organizmih je nujen ustrezno delujoč hormonski sistem, ki zagotavlja usklajeno delovanje hormonov. Ti delujejo kot kemični prenašalci sporočil med organi in tkivi. Hormone izločajo žleze z notranjim izločanjem, ti se nato prenesejo do ciljnih celic, kjer se vežejo na celične receptorje. Za normalno delovanje organizma je potrebno usklajeno delovanje številnih hormonov, ki delujejo sinhrono, v pravih koncentracijah in ob pravem času. Na hormonski sistem lahko vplivajo snovi, ki motijo njegovo delovanje na različne načine; lahko spreminjajo sintezo, transport, metabolizem hormonov in/ali tekmujejo za vezavo na celičnih receptorjih oz. spreminjajo nivo receptorjev na ciljnih celicah. Spremembe lahko vključujejo povečano ali zmanjšano aktivnost ali spodbujanje aktivnosti v neprimernih časih. Te snovi imenujemo hormonski motilci (angl. *endocrine disruptors*). Nekateri hormonski motilci so prisotni v naravi in so rastlinskega izvora (fitoestrogeni), druge proizvajajo glive (mikoestrogeni) (10, 11). Naravne hormonske motilce poznamo že dolgo časa. Prvi podatki o mikoestrogenih so se v literaturi pojavili že leta 1920, ko so na farmah prašičev v ZDA poročali o zmanjšanju reproduktivne sposobnosti svinj, ki so bile krmljene s plesnivim žitom (12). Kasneje so ugotovili, da je razlog za zmanjšanje reproduktivne sposobnosti svinj uživanje mikoestrogenov, ki jih vsebuje plesen (13). Drugi hormonski motilci, ki so javnosti postali bolje znani šele sredi devetdesetih let prejšnjega stoletja, so narejeni sintetično in jih zaradi tega natančneje imenujemo kemični povzročitelji hormonskih motenj (KPHM) (2). Med te spadajo zelo različne antropogene snovi, kot so sestavine plastike, detergentov in goriv, konzervansi, obstojna organska onesnaževala, pesticidi in produkti gorenja (11).

1.1.1 Kemični povzročitelji hormonskih motenj

KPHM je glede na definicijo Svetovne zdravstvene organizacije iz leta 2002 od zunaj vnesena snov oziroma zmes snovi, ki vpliva na delovanje hormonskega sistema in povzroči škodljive učinke na zdravje intaktnega organizma, njegovega potomstva oziroma (sub)populacije. Skladno z definicijo je za uvrstitev snovi med KPHM potreben dokaz vzročne povezave med izpostavljenostjo intaktnega organizma vneseni snovi in škodljivim učinkom po hormonskem načinu delovanja (2). Podobne definicije so podale tudi druge nacionalne in mednarodne organizacije, kot so Nemški zvezni urad za oceno tveganja (nem. *Das Bundesinstitut für Risikobewertung*; BfR), Evropska komisija (angl. *European Commission*; EC), Evropska

agencija za varnost hrane (angl. *European Food Safety Agency*; EFSA) in Ameriška agencija za varstvo okolja (angl. *Environmental Protection Agency*; EPA). Iz vseh definicij izhaja, da mora KPHM povzročiti škodljive učinke, kot so morfološke in funkcionalne spremembe celic, tkiv, organa ali organizma (14-17). Trenutno je na seznamu v Evropi priznanih KPHM, ki so ga leta 2020 na spletni strani edlists.org objavile nekatere evropske države (Belgija, Danska, Francija, Nizozemska, Švedska in Španija), več kot sto substanc, med njimi tudi BPA (18).

1.1.2 Prežvekovalci kot model za preučevanje KPHM

Za preiskovanje KPHM so bili ustvarjeni protokoli, na podlagi katerih se sprejemajo regulatorne odločitve. V protokolih je skoraj ekskluzivno predpisana raba *in vitro* metod ali laboratorijskih glodavcev, rezultate pa uporabljajo za oceno tveganja za ljudi. Laboratorijski glodavci se v raziskavah uporabljajo zaradi relativno nizke cene, kratkih razvojnih obdobij ter številnih in velikih legel (19).

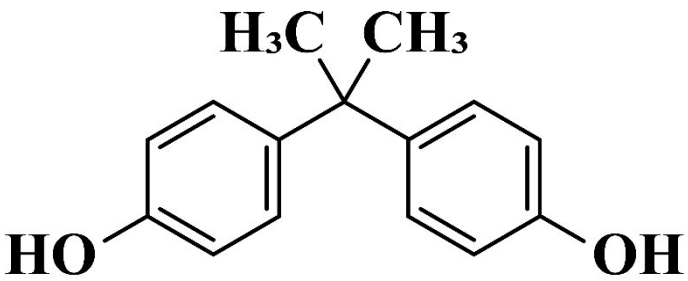
Pri delu z laboratorijskimi glodavci so se pokazale tudi nekatere omejitve, kot sta majhnost živali in s tem neprimernost za odvzem večjih količin vzorcev. Poleg tega sta interpretacija in ekstrapolacija rezultatov zaradi razlik v fiziologiji glodavcev in ljudi lahko zelo zahtevni ali celo nemogoči.

Raziskovalce je obenem zanimalo, kako splošne ali edinstvene so ugotovitve o KPHM pri laboratorijskih glodavcih, zaradi česar so se odločili za uporabo drugih modelnih organizmov, med katere spadajo prašiči (20), psi (21), primati (22), koze (23) in ovce (19, 24, 25). Nekatere druge raziskovalce je zanimalo, ali lahko KPHM v okolju, vključno s tistimi v krmi, vplivajo na reprodukcijsko zmogljivost rejnih živali (26, 27). Zaenkrat ni dovolj dokazov, da bi KPHM v neeksperimentalnih pogojih vplivali na reprodukcijo domačih živali (27).

1.1.3 Bisfenol A

1.1.3.1 Kemijske lastnosti

Bisfenol A (2,2'-bis(4-hidroksifenil)propan ali 4,4'-izopropildendifenol, BPA) s CAS številko 80-05-7 je organska snov in spada med alkilfenole. Je zmerno hidrofoben (logP 3,25), slabo topen v vodi (89 mg/l) in izraža šibke kisle lastnosti (pKa = 9,7) (Slika 1) (28). Njegova molska masa znaša 228,29 g/mol (29).



Slika 1: Kemijska struktura BPA

Figure 1: Chemical structure of BPA

1.1.3.2 Zgodovina in uporaba

BPA so po podatkih iz literature prvič sintetizirali že leta 1891 (30), vendar so ga šele v šestdesetih letih prejšnjega stoletja v industriji začeli uporabljati za proizvodnjo epoksi smol in polikarbonatne plastike (31). Ker je polikarbonatna plastika, ki vsebuje BPA, odporna na lomljenje in toploto ter je prozorna (32), je povpraševanje po BPA hitro naraščalo in še vedno narašča. Količina proizvedenega BPA naj bi samo v letu 2020 znašala 595307 ton (33). BPA se večinoma uporablja pri izdelavi polikarbonatih plastičnih materialov in epoksi smol, v manjši meri tudi pri proizvodnji zaviralcev gorenja, nenasičenih poliestrskih smol, polivinilklorida, poliakrilata in polisulfonskih smol. Najdemo ga v številnih izdelkih, kot so embalaža za hrano in pijačo, medicinski pripomočki, vodovodne cevi, barve, talne obloge, izolacije, tesnila, elektronika, tekstil, ogrevalni sistemi, pohištvo, papir, pesticidi, kozmetika, igrače in zobne plombe (29).

Pred uporabo v industriji so iskali možnosti uporabe BPA v farmaciji. Leta 1934 sta Dodds in Lawson preizkušala sintetične snovi, ki bi lahko nadomestile drag naravni estrogen, in s pomočjo testa na podganah ugotovila, da ima BPA šibko estrogeno delovanje (34, 35). BPA je tako tudi ena izmed prvih poznanih sintetičnih snovi, ki posnema delovanje ženskega spolnega hormona estrogena. Ker sta Dodds in Lawson s sodelavci v tem obdobju odkrila tudi farmakološko aktivnejšo snov dietilstilbestrol (DES), se je za nekaj desetletij v farmacevtske namene obdržala zgolj slednja (36).

1.1.3.3 Viri izpostavljenosti

Prisotnost BPA v naravnem okolju je povezana z antropogeno aktivnostjo, saj BPA v naravi ne nastaja. Največ BPA se v okolje izloči ob njegovi proizvodnji in obdelavi ter ob degradaciji polimerov (epoksi premazov in polikarbonatov). BPA iz polikarbonatov izhaja v večji meri s

hidrolizo polimerov, v manjši pa z difuzijo (37). Čeprav v okolju hitro razpada, se vanj neprestano izloča (38). Do danes so ga zaznali v hrani in pijači, vodi, tleh in celo v ozračju (39). Izpostavljenost ljudi in živali BPA je velika; rezultati raziskave Centra za nadzor bolezni (angl. *Center for Disease Control and Prevention*; CDC), ki je bila izvedena na več kot 2500 prebivalcih ZDA, so pokazali, da so v urinu pri več kot 92,6 % zaznali BPA (40). Prav tako so BPA zaznali v mleku doječih mater (41-47).

Ljudje so BPA pretežno izpostavljeni z uživanjem hrane in pijače. Viri vnosa BPA pa so tudi zrak, prah, izdelki za osebno nego in termični papir (48, 49). Vnos je tako v manjši meri mogoč tudi z vdihavanjem ali skozi kožo (50).

V literaturi nismo zasledili podatkov, v kolikšni meri so BPA izpostavljene živali. Gorecki in sod. so analizirali surovo meso rejnih živali in v njem ugotovili zgolj prosti BPA, ne pa drugih metabolitov monoglukuronida, diglukuronida in sulfata, ki bi nakazovali, da so bile rejne živali izpostavljene BPA (51). Pri maloštevilnih analizah hrane psov in mačk ter krme za živali (52-56) so ugotovili, da lahko živali s hrano zaužijejo BPA. V mokri hrani za pse v konzervah so namreč namerili 11–206 ng/g BPA (53) in 5–208 ng/g BPA (55), v mačjih konzervah 13–136 ng/g BPA (53), v mokri mačji hrani 1,58 ng/g BPA, v vrečkah z mokro mačjo hrano 0,591 ng/g in v suhi mačji hrani 1,18 ng/g BPA (54). Na Kitajskem so ugotovili, da BPA prehaja tudi iz plastične embalaže v krmo za živali, saj so ga namerili v koncentracijah od 6,24 do 2443 ng/g BPA, odvisno od vrste plastične embalaže (52).

1.1.3.4 Regulatorni vidiki

Zaradi vseprisotnosti, široke uporabe in skrbi za zdravje ljudi je BPA v delih sveta reguliran na nekaterih področjih uporabe in izdelkov (57).

Evropska unija

EFSA je kmalu po ustanovitvi leta 2006 določila vrednost sprejemljivega dnevnega vnosa[1] (angl. *tolerable daily intake*; TDI) BPA na 50 µg BPA/kg t. m. Vrednost TDI je bila določena z uporabo 100-kratnega faktorja glede na vrednost najvišjega odmerka, pri katerem ni prišlo do opaznih neželenih učinkov (angl. *no-observed-adverse-effect level*; NOAEL). EFSA je svojo oceno tveganja za BPA posodobila v letih 2008 in 2010 in obakrat potrdila vrednost TDI iz leta

[1] TDI je ocena količine onesnaževalca v hrani ali pitni vodi, izražena na podlagi t. m., ki se lahko zaužije vsak dan vse življenje brez znatnega tveganja za zdravje potrošnika na podlagi vseh znanih dejstev v času ocene (58).

2006 (59-61) ter leta 2010 podala mnenje, da je količina vnesenega BPA do te vrednosti varna ter da je verjetno izpostavljenost vseh skupin prebivalstva pod to mejo. Kljub temu je EFSA izrazila nekaj dvomov glede možnega vpliva BPA na dojenčke in ugotovila, da so glede tega potrebni zanesljivejši podatki (60, 61). Na podlagi tega mnenja je zaradi previdnosti januarja 2011 EC prepovedala uporabo BPA v plastičnih stekleničkah za hranjenje dojenčkov v EU (62). Leta 2015 je EFSA na podlagi novih podatkov in metod za BPA začasno zmanjšala vrednost sprejemljivega dnevnega vnosa (angl. *temporary TDI*; t-TDI) na 4 µg/kg t. m. do pridobitve rezultatov dalj časa trajajočih raziskav na podganah, ki so bile BPA izpostavljene prenatalno in postnatalno. Na podlagi Znanstvenega mnenja o tveganjih za javno zdravje glede prisotnosti bisfenola A v živilih iz leta 2015 so zaključili, da je izpostavljenost BPA po dietarnem in nedietarnem vnosu pod dovoljeno mejo, in sicer dva- do petkrat nižja od vrednosti t-TDI, odvisno od starostne skupine (63). V osnutku ponovne ocene BPA, napisanem 15. 12. 2021, je strokovni svet EFSA za materiale v stiku z živili, encime in pripomočke za predelavo predlagal novo vrednost TDI 0,04 ng/kg t. m./dan. V osnutku so zapisali, da je znižanje vrednosti TDI predvsem posledica rezultatov raziskav na živalih v obdobju med letoma 2013 in 2018, ki so pokazale na škodljive učinke BPA na imunski sistem. V raziskavah na živalih so opazili povečanje števila T celic pomagalk, ki igrajo ključno vlogo pri celičnih imunskih mehanizmih in ki lahko povzročijo alergijsko vnetje pljuč (64).

Druge države

Kanada je bila prva država, v kateri so omejili uporabo, uvoz, oglaševanje in prodajo izdelkov, ki vsebujejo BPA. Ta omejitev se je nanašala na polikarbonatne otroške stekleničke, pločevinke in drugo embalažo, v kateri je bila otroška hrana (65).

V ZDA je Uprava za hrano in zdravila (angl. *Food and Drug Administration*; FDA) junija 2012 prepovedala uporabo BPA v otroških stekleničkah in skodelicah. Nato je junija 2013 prepovedala uporabo BPA v embalaži za pakiranje živil, ki se uporabljajo za otroško prehrano. FDA je leta 2008 za BPA določila vrednost NOAEL za peroralni vnos na 5 mg/kg t. m./dan (66), na podlagi česar so postavili vrednost TDI 50 µg/kg t. m./dan, ki velja še danes (67).

V državah Afrike, JV Azije ter Južne in Srednje Amerike, ki spadajo med države v razvoju, omejitev uporabe BPA skoraj ni (68).

1.1.3.5 Analogi

Omejevanje uporabe BPA je privedlo do iskanja alternativnih snovi. Namesto BPA se zaradi strukturne podobnosti najpogosteje uporabljata bisfenol F (BPF) in bisfenol S (BPS) (69, 70). Po podatkih Evropske agencije za kemikalije (angl. *European Chemical Agency*; ECHA) se v Evropi letno proizvede ali uvozi 1000–10000 milijonov ton BPS (71) in 10–100 ton BPF (72). BPS in BPF so dokazali v izdelkih za osebno nego (73), v izdelkih iz papirja in v živilih (69, 70). BPS se uporablja v čistilnih izdelkih, premazih za električne izdelke in različnih industrijskih aplikacijah kot sestavina fenolnih smol, v termičnem papirju, vključno z izdelki, ki se tržijo kot »papir brez BPA«. BPF je prisoten v vodovodnih ceveh, zobnih tesnilnih masah, premazih za rezervoarje in cevi, industrijskih tleh, premazih za ceste in mostove, strukturnih lepilih in malti. Poleg tega se uporablja tudi v embalaži za živila. BPF je tudi sestavni del epoksidnih smol (69, 70). Zaradi vse večje uporabe in strukturne podobnosti z BPA sta BPS in BPF pritegnila pozornost raziskovalcev, ki so ugotovili, da imajo lahko tudi analogi BPA škodljive učinke za zdravje, saj so lahko citotoksični, genotoksični in mutageni ter imajo lahko toksične učinke na reproduktivne organe (74-77).

1.1.4 Metode za detekcijo BPA v bioloških vzorcih

Določitev vsebnosti BPA v bioloških vzorcih vključuje naslednje korake: zbiranje in hranjenje vzorcev, pripravo vzorcev, ki vključuje predpripravo, ekstrakcijo in čiščenje vzorcev ter določitev koncentracije z inštrumentalno analizno metodo (28).

1.1.4.1 Priprava vzorcev

Za določanje BPA v bioloških vzorcih je ključen korak priprava vzorcev, ki je večinoma dolgotrajna in delovno intenzivna (78, 79). Priprava vzorcev je v veliki meri odvisna od vrste vzorca. Za predpripravo trdnih vzorcev se običajno uporablja homogenizacija, za predpripravo tekočih vzorcev pa se uporabljata filtracija in/ali centrifugiranje. Poleg tega je za posebne vzorce potrebna posebna predpriprava, npr. vzorci z visoko vsebnostjo beljakovin lahko zahtevajo njihovo odstranitev z obarjanjem (28).

Za ekstrakcijo in čiščenje BPA v bioloških vzorcih se uporabljajo različni pristopi, najpogosteje so to ekstrakcija s topilom (angl. *solvent extraction*; SE), ekstrakcija tekoče-tekoče (angl. *liquid-liquid extraction*; LLE) in ekstrakcija na trdni fazi (angl. *solid phase extraction*; SPE). Drugi pristopi so še ekstrakcija s pomočjo mikrovalov (angl. *microwave-assisted extraction*,

MAE), tekočinska ekstrakcija pod visokim pritiskom (angl. *pressurized liquid extraction*; PLE), disperzija trdne faze v matriksu (angl. *matrix solid-phase dispersion*; MSPD), mikroekstrakcija na trdnem nosilcu (angl. *solid-phase microextraction*; SPME) in sorptivna ekstrakcija z mešalnimi palicami (angl. *stir bar sorptive extraction*; SBSE) (28). Od leta 2000 so se metode priprave vzorcev precej razvile, k temu sta v veliki meri prispevali tako uporaba nanostrukturnih tekočin, imenovanih supramolekularna topila, ki združujejo ekstrakcijo in čiščenje v eni fazi (80, 81), kot tudi uporaba selektivnih sorbentov, kot so molekularno vtisnjeni polimeri (angl. *molecularly imprinted polymers*; MIPs) (Slika 2). To so sintetični, po meri izdelani polimeri z vnaprej določeno strukturno selektivnostjo za določen analit ali skupino strukturno povezanih spojin. Nosilce MIP dobimo s polimerizacijo funkcionalnih in zamreženih monomerov okoli vzorčne molekule, pri čemer nastane močno zamrežen tridimenzionalni mrežni polimer (82, 83). Nastali vtisnjeni polimeri so zelo stabilni, robustni in odporni proti fizikalno-kemijskim spremembam, kot so sprememba vrednosti pH, temperature in topil, zato tehnika molekularnega vtiskovanja predstavlja obetavno in ugodno alternativo za premagovanje težav, povezanih z biomolekulami, kot so protitelesa, encimi in druge receptorske molekule (84). Nosilci MIP lahko omogočijo izolacijo in čiščenje analitov v enem koraku ter zagotovijo čistejše ekstrakte v primerjavi s klasično SPE (28). Uporaba MIP v ekstrakciji na trdni fazi oz. tako imenovana ekstrakcija na trdni fazi z molekularnim odtisom (MISPE) je daleč najnaprednejša uporaba MIP (79).

Slika 2: Ekstrakcijska kolona za ekstrakcijo na trdni fazi s selektivnim polnilom z MIP tehnologijo

Figure 2: Solid phase extraction column with selective filler with MIP technology

1.1.4.2 Določitev koncentracije z inštrumentalno metodo

Za določitev koncentracije BPA v kompleksnih bioloških vzorcih je potrebna uporaba visoko občutljivih in selektivnih analiznih metod. Zato se za določanje koncentracije BPA v slednjih uporabljajo predvsem kromatografske metode, in sicer najpogosteje tekočinska kromatografija visoke ločljivosti s fluorescenčno detekcijo (HPLC-FL), tekočinska kromatografija visoke ločljivosti s tandemsko masno spektrometrično detekcijo (HPLC-MS/MS) in plinska kromatografija s tandemsko masno spektrometrično detekcijo (GC-MS/MS).

Tekočinska kromatografija je v primerjavi s plinsko kromatografijo enostavnejša, saj zanjo ni potrebna visoka temperaturna obstojnost analitov oz. ni potreben dodaten korak derivatizacije vzorcev, vendar plinska kromatografija zaradi dolgih kromatografskih kolon običajno omogoča boljšo resolucijo. Druge tehnike, kot so tekočinska kromatografija visoke ločljivosti z elektrokemično detekcijo, kapilarna elektroforeza (angl. *capillary electrophoresis*), imunoanaliza (ELISA in imunokromatografske metode), in nekatere nove metode, kot so senzorji, se uporabljajo v manjši meri (28, 78).

Tekočinska kromatografija visoke ločljivosti s fluorescenčno detekcijo

HPLC-FL se zaradi svoje visoke specifičnosti in selektivnosti šteje za primerno potrditveno metodo za organske ostanke ali kontaminante, ki kažejo nativno fluorescenco, in za molekule, ki kažejo fluorescenco po kateri koli transformaciji ali derivatizaciji (85). Nativno fluorescenco z vzbujevalno in emisijsko valovno dolžino 275 oziroma 305 nm kaže tudi BPA, kar smo uporabili tudi pri naših meritvah. Običajne inštrumentalne meje za kvantifikacijo BPA s to metodo so v območju med 5 in 50 ng/ml. Metoda je tako primerna za določanje BPA v zelo različnih vzorcih. Uporabili so jo že za določanje BPA v pijači, vinu, hrani za živali, sadju, zelenjavi, medu in ribah (53, 86-91). Običajne meje detekcije za določanje BPA v hrani in pijači so bile med 0,1 in 2 ng/ml oz. med 1 in 5 ng/g (28).

1.1.5 Toksikokinetika BPA

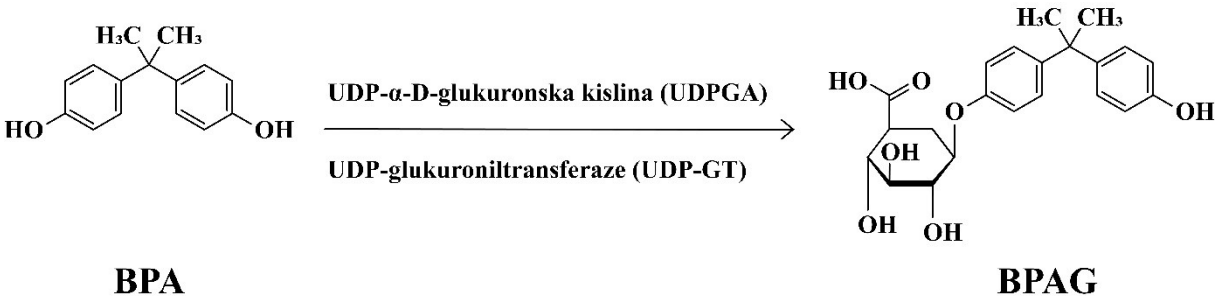
TK je veda, ki proučuje vplive organizma na toksin, torej vplive na njegovo sprostitev, absorpcijo, presnovo v telesu in izločanje iz telesa. Določitev absorpcije, porazdelitve v tkivih, metabolizma in izločanja BPA so prvi pogoj za oceno tveganja, saj so ti parametri temeljni pri ugotavljanju toksičnosti. Ti procesi so odvisni od načina vnosa BPA v organizem (92).

1.1.5.1 Absorpcija in metabolizem

Pri ljudeh, primatih in glodavcih se BPA po peroralnem vnosu v veliki meri in hitro absorbira iz gastrointestinalnega trakta (93-97); v raziskavi Völkla in sod. se je po peroralnem vnosu z urinom izločilo 97 % vnesenega odmerka pri moških in 84 % vnesenega odmerka pri ženskah (93, 94). Rezultati raziskav na ljudeh so sovpadali z rezultati raziskav na opicah (95).

Po peroralni aplikaciji je absolutna biološka uporabnost pri primatih 0,9-1,9 %, pri podganah pa 2,8 % (98). BPA se iz gastrointestinalnega trakta prenese v jetra, kjer se s pomočjo jetrnih encimov pretvori v metabolite (3).

Glavni metabolit, ki nastane z mehanizmom konjugacije z glukuronsko kislino pod vplivom encimov UDP-glukuroniltransferaz, je BPAG (Slika 3) (93, 99).



Slika 3: Reakcija konjugacije aglikona BPA v BPAG

Figure 3: Conjugation reaction of aglycone BPA to BPAG

Poleg glukuronidiranja je eden od procesov metabolizma BPA tudi sulfatiranje, ki poteka z encimi sulfotransferazami (100). Sulfatiranje je manj pomembna metabolna pot (101) in pri primatih predstavlja manj kot 20 %, pri podganah pa manj kot 5 % metabolizma BPA (61). Pri ljudeh so v raziskavi, izvedeni na 30 prostovoljcih, ugotovili, da je BPAG v urinu predstavljal 69,5 % vnesenega odmerka, z 21 % mu je sledil BPA-sulfat, najmanj (9,5 %) pa je bilo prostega BPA (102). Provencher in sod. pa so v nedavni raziskavi na prostovoljcih ugotovili, da se je z urinom v obliki BPAG izločilo 94,6 % odmerka BPA, v obliki BPA-sulfata 3,7 % odmerka in v obliki prostega BPA 1,7 % odmerka (103).

Poleg BPAG in BPA-sulfata nastanejo pri visokih koncentracijah vnesenega BPA še nekateri drugi metaboliti, in sicer 5-hidroksi BPA, 3-hidroksi BPA in BPA-O-kinon. Do njihovega nastanka pride ob nasičenju encimov, ki katalizirajo reakcijo glukuronidacije (104).

BPAG in BPA-sulfat nista več estrogensko aktivna, medtem ko so za oksidativne metabolite BPA v *in vitro* raziskavah ugotovili enako estrogensko aktivnost kot pri prostem BPA (101).
V primerjavi s peroralnim vnosom je biološka uporabnost BPA večja pri parenteralnih vnosih, kot so podkožni, intravenski in intraperitonealni vnos (105). Raziskovalci menijo, da peroralni vnos s sondo ni enak dietarnemu vnosu (106). Dietarni vnos BPA so uporabili v raziskavi s psi (21), primati (107), na ljudeh (108, 109) ter v eni raziskavi z ovcami (5), kjer so ugotovili, da se TK parametri razlikujejo glede na način vnosa. Pri dietarnem vnosu se BPA absorbira skozi sluznico ustne votline in iz želodca ter črevesja, medtem ko se pri peroralnem vnosu s kapsulami ali sondo absorbira le v prebavnem traktu. Biološka uporabnost BPA po dietarnem vnosu je bila prav tako večja v primerjavi s peroralnim vnosom s sondo (21).

1.1.5.2 Izločanje

Pri ljudeh in primatih se BPAG zaradi povečane hidrofilnosti izloča predvsem skozi ledvice z urinom (61), razpolovni čas ($t_{1/2}$) pri ljudeh je manj kot 6 ur (108). Pri glodavcih se BPAG izloča v žolč in iz žolča v črevo, kjer se iz črevesja v krvni obtok reabsorbira v prosti obliki (enterohepatični cikel). Opisani cikel se večkrat ponovi. Izločanje BPA pri glodavcih v večji meri poteka z iztrebki. Pri podganah je $t_{1/2}$ med 19 in 78 urami. Zaradi počasnega izločanja so glodavci dlje časa izpostavljeni prostemu BPA (110).

Izločanje v mleko

Raziskave, v katerih so preučevali prenos BPA v mleko, so zelo redke. Vse *in vivo* raziskave so bile opravljene na glodavcih (111-114).
Doerge in sod. so raziskovali prenos BPA v mleko podgan po ponavljajočem peroralnem vnosu in ugotovili 0,19 µg/l prostega BPA in 1,6 µg/l celokupnega BPA eno uro po vnosu 100 µg BPA/kg t. m. Na podlagi rezultatov so izračunali, da mladiči z mlekom prejmejo zgolj 1/300 odmerka, ki so ga dali materam (113). Da bi se raziskovalci izognili kontaminaciji vzorcev zaradi vsesplošne razširjenosti BPA v okolju, so v raziskavi uporabili z izotopom označen BPA. Odmerek 100 µg/kg t. m. je bil izbran zato, da je bil znotraj linearnega TK območja in da je bil čim bližje območju predlagane izpostavljenosti ljudi, vendar dovolj visok za merjenje proste in konjugirane oblike. Raziskovalci menijo, da je prenos BPA v mleko omejen s serumskimi ravnmi prostega BPA, čeprav se lipofilna oblika prostega BPA učinkovito porazdeli v mleko (koeficient porazdelitve mleko/serum je 1,3). Ta vrednost je podobna razmerju mleko/serum

vrednosti 2,5, o kateri so poročali v raziskavi Yoo in sod., kjer so podganam namestili intravensko infuzijo in jim BPA vnašali do stacionarnega stanja v krvni plazmi (111). Koncentracije prostega BPA v raziskavi Doerge in sod. (113) so bile podobne koncentracijam (2000 nM) iz raziskave Okabayashija in Watanabeja, ki so jih v mleku izmerili 2 uri po peroralnem vnosu BPA podganam Sprague–Dawley (112). V raziskavi so Doerge in sod. glede na porazdelitveni koeficient, ki je bil manjši za konjugirano kot za prosto obliko BPA, domnevali, da konjugirana oblika BPA slabo prehaja v mleko (porazdelitveni koeficient = 0,062) (113). Podobno vrednost (0,06) bi dobili iz meritev skupne radioaktivnosti v mleku in krvni plazmi pri podganah F344 v laktaciji eno uro po peroralnem vnosu 500 μg ^{14}C-BPA/kg t. m. (115).

1.1.6 Toksikodinamika BPA

Zaradi visoke proizvodnje, uporabe v širokem razponu izdelkov in posledične razširjenosti v okolju ter izpostavljenosti ljudi je BPA danes eden izmed najbolj raziskanih KPHM (116).

BPA se lahko veže na oba estrogenska receptorja: na estrogenski receptor alfa (Erα) in beta (Erβ) s tem, da je njegova afiniteta do Erβ večja (117). Njegova estrogenska aktivnost je približno pet velikostnih redov nižja od estrogenske aktivnosti 17β-estradiola (118, 119). BPA v *in vitro* testih kaže tudi antiandrogensko delovanje (101, 120). Prav tako je opisano njegovo delovanje na pregnanske X receptorje (PRX) (35), estrogenskemu receptorju podobne receptorje gama (ERRγ) (36), aril ogljikovodikove receptorje (AhR) (121), na membranske estrogenske receptorje ter s proteinom G sklopljene receptorje 30. BPA je tudi antagonist receptorju za ščitnične hormone (TR) (57, 122).

Iz navedenih rezultatov raziskav izhaja, da BPA lahko deluje na različne organske sisteme in tkiva. V raziskavah so ugotovili, da lahko vpliva na presnovo in na imunski, živčni in reproduktivni sistem (123).

1.1.6.1 Vpliv na moške spolne organe

Vpliv BPA na moške spolne organe so ugotavljali številni raziskovalci v epidemioloških, *in vitro* ter in *vivo* raziskavah.

Epidemiološke raziskave

V epidemiološki raziskavi, opravljeni na Kitajskem, so ugotovili, da so imeli delavci, ki so bili izpostavljeni visokim dozam BPA na delovnem mestu v proizvodnji BPA, manjši libido in večje težave z erekcijo in ejakulacijo (124). V naslednji raziskavi so ugotovili, da imajo moški z višjimi koncentracijami BPA v urinu nižjo koncentracijo semena, nižji odstotek gibljivih semenčic in nižji odstotek vitalnih semenčic (125). Meeker in sod. so med preučevanjem neplodnih parov ugotovili, da sta večja fragmentacija DNA semenčic in manjša kvaliteta semena povezani z višjo koncentracijo BPA v urinu teh moških (126). Po drugi strani Goldstone in sod. (127) ter Mendiola in sod. (128) niso ugotovili nobene povezave med višjimi vrednostmi BPA in reprodukcijskimi parametri.

In vitro raziskave

In vitro raziskave, pretežno opravljene na celičnih kulturah Sertolijevih celic podgan, so pokazale, da BPA negativno vpliva na njihovo vitalnost in celo povzroča apoptozo (129). Wang in sod. menijo, da je apoptoza Sertolijevih celic, ki so bile izpostavljene BPA, posledica delovanja BPA na PTEN/AKT signalno pot (130). Medtem so Qi in sod. dokazali, da BPA lahko povzroči apoptozo Sertolijevih celic z aktivacijo JNK/p38 MPAK signalne poti in da translokacija NF- κB in Fas/FasL sistema igra ključno vlogo v sproženju apoptoze (131). Poleg tega so Wang in sod. ugotovili, da po izpostavitvi podganjih Sertolijevih celic BPA (50 in 70 μM na dan) pride do povečanega nastanka reaktivnih kisikovih zvrsti (angl. *reactive oxygen species*; ROS) in disfunkcije mitohondrijev (132). Barbonetti in sod. so enak rezultat dobili, ko so seme moških izpostavili BPA v koncentraciji 300 μM (133).

In vivo raziskave

V literaturi so najštevilčnejše prav *in vivo* raziskave na živalih v postopkih, med katerimi prevladujejo raziskave na glodavcih. Kljub temu da velja, da so zarodki, novorojenčki in dojenčki najbolj občutljivi za škodljive učinke BPA (134), so bile nekatere raziskave opravljene tudi na glodavcih v kasnejših obdobjih življenja, v puberteti in v odrasli dobi (135-160). V teh raziskavah so ugotovili zmanjšanje absolutne in/ali relativne mase mod in/ali nadmodkov (135-141) oz. povečanje mase mod (142), manjši premer zvitih semenskih cevk (136, 140, 143), nižjo višino zarodnega epitela zvitih semenskih cevk (142, 144, 145) ali večje število apoptotičnih celic (146-149). S histopatološko preiskavo so v modih ugotovili zmanjšanje

števila Leydigovih celic (136, 138), degeneracijo Leydigovih celic in zarodnih celic (141), zmanjšano število zarodnih celic (146, 147), odluščene zarodne celice (143, 150), nekrozo zarodnega epitela (151) in citoplazemsko vakuolizacijo zarodnih celic (145). V nekaterih raziskavah so ugotovili vpliv BPA na kakovost in količino semena, in sicer je BPA zmanjšal gibljivost semenčic v nadmodku, število semenčic, proizvodnjo semenčic, zaloge semena in tranzitni čas semena ter povzročil poškodbe DNK semenčic (135, 145, 152-155). Poleg tega je BPA porušil prooksidativno-antioksidativno ravnovesje v tkivu mod in nadmodka odraslih podgan (135, 137, 153, 156). Eden izmed razlogov za različne rezultate opisanih raziskav je lahko uporaba protokolov raziskav, ki so si bili med seboj zelo različni. V raziskavah so bili uporabljeni odmerki v širokem razponu (0,0002 do 960 mg/kg t. m. (135, 136), v različnih časovnih izpostavljenostih (od 6 dni do 48 tednov) (142, 150) in pri različnih načinih vnosa (pitna voda, dajanje v žrelo, podkožno, intraperitonealno) (144, 150, 152, 157). Poleg tega so raziskovalci uporabili različne soje podgan (Wistar, Sprague-Dawley, albino) (137, 144, 153) in mišk (Kunming, Pzh: SFIS, CD-1 miške, švicarsko-albinske miške, ICR miške, C57BL/6 miške) (146, 147, 150, 158-160), ki so prav tako različno dovzetne za učinke estrogenih snovi (7).

Le dve *in vivo* raziskavi sta bili izvedeni na drugih vrstah sesalcev, na navadnih marmozetkah (*Callithrix jacchus*) (22) in kozah (*Capra hircus*) (161). Navadne marmozetke so v omenjeni raziskavi prejemale BPA v odmerku 2,5, 12,5 ali 25 µg/kg t. m./dan. V skupini opic, ki je prejemala 12,5 µg/kg t. m. BPA/dan, so ugotovili povečano število odluščenih zarodnih celic v lumnih zvitih semenskih cevk in edem intersticija; v skupini opic, ki je prejemala 25 µg/kg t. m. BPA/dan, pa so ugotovili vakuolizacijo Sertolijevih celic in zmanjšanje števila primarnih spermatocitov in okroglih ter podolgovatih spermatid (22). Odrasli kozli so BPA prejemali v odmerku 25 mg/kg t. m./dan, BPA je bil v tej raziskavi uporabljen za pozitivno kontrolo, glavna preučevana snov pa je bila rastlinski izvleček lepega slaka (*Ipomoea carnea*). Edini ugotovljeni spremembi pri kozlih, izpostavljenih BPA, sta bili vakuolarna degeneracija celic rete testis in zmanjšana integriteta celične membrane semenčic (161).

1.2 HIPOTEZE

Zastavili smo si tri delovne hipoteze:

1. V vzorcih mleka ovce je BPA mogoče zaznati po dietarnem in podkožnem vnosu.
2. Način vnosa in fiziološko obdobje živali vplivata na TK profil BPA.
3. 64-dnevna izpostavljenost BPA s hrano, v odmerku 25 µg/kg t. m., vpliva na morfološke značilnosti mod in osnovne parametre semena.

2 OBJAVLJENA ZNANSTVENA DELA

2.1 PRELIMINARNA TOKSIKOKINETIČNA RAZISKAVA PRI MLEČNI OVCI V LAKTACIJI PO DIETARNEM IN PODKOŽNEM VNOSU BISFENOLA A

Preliminary toxicokinetic study of BPA in lactating dairy sheep after repeated dietary and subcutaneous administration

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Izvleček

Prehrana je glavni način izpostavljenosti ljudi BPA, eno od pomembnejših živil je mleko. Namen naše preliminarne raziskave je bil ovrednotiti izpostavljenost BPA in njegovo razpoložljivost v ovčjem mleku po večkratnem dietarnem in podkožnem vnosu relativno nizkega dnevnega odmerka (100 µg/kg t. m./dan) ovci v postopku. Z razvojem toksikokinetičnega modela smo na podlagi meritev krvne plazme in mleka ocenili odstotek izločenega BPA v mleko, ki je bil manjši od 0,1 % ne glede na način vnosa. Na podlagi toksikokinetičnega modela smo tudi ugotovili, da se BPA pri ovcah iz krvi v mleko verjetno prenese v večji meri v prosti (nekonjugirani) obliki. Na podlagi te ugotovitve in izvedene meritve domnevamo, da se prosti BPA verjetno naknadno presnovi v mlečni žlezi. Odstotek odmerka BPA, izločenega v mleko, je minimalen ne glede na način vnosa ovci v postopku.

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Preliminary toxicokinetic study of BPA in lactating dairy sheep after repeated dietary and subcutaneous administration

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Dietary intake is the predominant route of human exposure to bisphenol A and one of the important food commodities is milk. The aim of our study was to preliminarily evaluate the bisphenol A exposure and disposition in sheep milk after repeated dietary and subcutaneous administration of a relatively low dose (100 µg/kg of b. w./day) of bisphenol A to a sheep. On the basis of blood plasma sampling, milk sampling and HPLC analysis, we developed the toxicokinetic model. With the toxicokinetic model we showed that most likely only free bisphenol A passes into the mammary gland and is subsequently conjugated there. The percentage of the dose eliminated with milk was less than 0.1%, regardless of the route of bisphenol A administration. It is proven that the bisphenol A is eliminated through the milk of lactating sheep. However, the amounts excreted in the milk that were detected in this study are minimal.

Since the start of the commercial production of bisphenol A (BPA) in the 1950s until the present, the global production and consumption of this substance, regardless of the suspected negative health effects, has continued to rise[1]. With both the wide use of BPA and its leaching from many products and materials[2], it is known to be one of the ubiquitous environmental contaminants[3]. The main route of human BPA exposure is thought to be oral ingestion (up to 83% of the total estimated exposure), and in 2013 canned products accounted for about 50% of the dietary exposure to BPA. Thus, cans and packaging are believed to be the main source of contamination in foods[4]. Current migration limit of BPA from varnishes or coatings applied to materials and articles is 0.05 mg/kg BPA of food[5]. However, the products from farm animals, being directly exposed to human pollution, could still be, in some cases, an additional risk factor for human exposure[6].

It is believed that BPA causes endocrine disrupting effects by the interaction with various receptors, such as thyroid hormone receptor, androgen receptor and oestrogen receptor. Thus, BPA health hazards for reproductive system, nervous system, metabolic function, immune function, the growth and development of offspring were raised[4,7]. The European Food Safety Authority (EFSA) decreased the tolerable daily intake (TDI) from the 50 µg/kg of b. w./day to 4 µg/kg of b. w./day as a response to a refined risk assessment of BPA[7].

A food commodity important in our daily diet is milk. The quality and safety of milk depends considerably on the environment and human activity in its production. A broad range of environmental contaminants can enter the milk chain in the beginning via application of contaminated material on the soil such as industrial waste and sewage sludge, and atmospheric deposition from industrial activities[6]. It is also true that chemicals can enter milk during the collection and preparation processes of dairy products[8]. For instance, BPA may be introduced during milking from plastic parts of the milking machines, or also transferred from bulk milk to plastic storage tanks[9]. Finally, BPA can migrate as an additive from packaging material into the consumable milk. The actual levels of BPA found in commercial milk samples are presented in the review of Mercogliano and Santonicola, and are in the range between not detected (ND) to 521 ppb[6].

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To the best of our knowledge, only a few *in vivo* studies are published regarding BPA transfer to milk, with all of them using rodent models, and all report limited excretion of BPA into milk[10–13]. Doerge *et al.* evaluated the lactational transfer of BPA after repeated oral dosing in rats, and found concentrations of 0.83 +/− 0.26 nM of free BPA and 7.6 +/− 2.8 nM of total BPA 1 hour after the administration of 100 µg/kg b. w.. They calculated that doses delivered to pups lactationally were 300-fold lower than the dose administered to the dams[13].

Cows are mainly used in milk production in Europe, accounting for 96.9% of the total milk produced[14]. However, a significant part of the agrarian economies and sheep dairy products have gained market size due to the product's quality, high yield, and high nutritional value[15]. Sheep are also frequently used as a model for cattle and other large mammals, due to their easier manipulation. The comparable digestive physiology (polygastric model) in sheep and cows enables the assumption that the sheep model is in terms of toxicokinetics (TK) a relevant model for cows as well[16].

The aim of our study was to estimate the transfer of BPA from feed or via subcutaneous administration to milk. To do so, one Slovenian autochthonous dairy sheep, an Istrian Pramenka, and her lamb were used in the study. Time courses of the free BPA, bisphenol A glucuronide (BPA-GLUC) and total BPA concentrations were followed in the ewe's blood plasma after repeated dietary and subcutaneous administration, as well as BPA transfer in milk. We also aimed to assess lactational transfer of BPA to the suckling lamb by estimating BPA exposure in its blood plasma. As gastrointestinal tract of our ruminant model is very specific and the detection of BPA or BPA-GLUC in the milk was never performed after *in vivo* experiment in this species, we were unable to predict the outcomes of the study. Thus, only one animal and only one concentration gradient (100 µg/kg b. w.) was used in this preliminary study.

Materials and methods

Chemicals. Bisphenol A ≥ 99% purity (Merck, Sigma-Aldrich, Darmstadt, Germany) was dissolved in absolute ethanol and corn oil for the dietary (po) and subcutaneous (sc) routes of administration, respectively. The volume administered to the sheep was adjusted to the body weight recorded on the day of the administration. For the dietary administration, approximately 1 mL of BPA solution in absolute ethanol was applied onto the pellet ration to obtain the single dose of 100 µg/kg b. w., and applied with the morning feed of pellets (400 g). For the sc administration, the injection of BPA solution was performed in the shoulder area (2.9 mL) at the same dose. Both solutions were stored at the ambient temperature in sealed amber glass bottles for the entire duration of use. All materials used for the solution preparation, sample processing and assays were either made of glass or of BPA-free plastics.

The dose of 100 µg/kg b. w. was chosen in this study. As it is within the linear pharmacokinetic range at a level as close as possible to the range of proposed human exposure, yet high enough to measure both aglycone (i.e., active) and conjugated (i.e., inactive) forms of BPA in samples analyzed[13].

Animal husbandry. All animal procedures were carried out in accordance with ethical standards and approved by the Administration of the Republic of Slovenia for Food Safety, Veterinary Sector and Plant Protection with permission no. U34401-3/2015/8. The study was performed on one stabled, healthy lactating Istrian Pramenka sheep with a single female suckling lamb in a sheepfold at the Infrastructure Centre for Sustainable Recultivation at Vremščica belonging to the Veterinary Faculty of the University of Ljubljana, Slovenia. The ewe was six years old and weighed 59 kg, while the suckling lamb was four weeks old and weighed 12 kg. The ewe and lamb were kept under natural temperature and photoperiodic conditions, with free access to water, hay and salt. In addition, the sheep was fed twice a day with 400 g plant based pellets (SchafKorn Lac, Unser Lagerhaus Warenhandels Ges., Austria). Eventual contamination of the experimental environment was checked by preliminary testing of drinking water and pellets by high-performance liquid chromatography (HPLC) analysis, which revealed the slight presence of BPA of 0.02 µg/L and 5 µg/kg in these two matrices, respectively. The sheep and its lamb were, at both periods of the study, penned individually the day before the first administration until three days after the last administration. The lamb was kept with its mother, except on sampling days, when they were separated for a few hours before sampling time to collect enough milk for analysis. The animals were clinically healthy, as indicated by medical (temperature, breathing and rumination frequency, pulse rate) (see Supplementary Table S1), haematological and biochemical (see Supplementary Tables S2 and S3) examinations. Fourteen days after the second experimental period, the sheep and its lamb were released in their original herd.

Experimental design. The experiment was divided into two periods, the first being the dietary administration period and the second being the subcutaneous administration period. The same ewe was used for both exposure routes. Before the start of the second period, a 13 days wash-out period was permitted to ensure that BPA was removed from the body of the ewe. In the first period, the ewe received BPA in its diet (100 µg/day/kg b. w.) for five consecutive days (dietary route of administration). The ewe ingested all pellets within 2–9 minutes. During the second period, the same ewe was injected in the shoulder area with 100 µg/kg b. w. of BPA subcutaneously per day for five consecutive days (subcutaneous route of administration).

On the first day of the dietary period of the experiment, the ewe's blood samples were taken at time 0 (before the first administration) and at 0.083, 0.16, 0.33, 0.5, 1, 2, 4, 6, 8, 10 and 24 hours after the first administration. The blood samples were then taken every day for the next seven days (trough concentrations). The sampling time started when the ewe ingested the whole portion of pellets. Similar sampling intervals were used in the second subcutaneous period of the experiment, with the exception of the first blood sampling (0.083 h after the sc administration), which was not taken. Blood samples from the suckling lamb were collected on the first day 10 hours after BPA administration to the ewe, and on every following day before the next administration to the ewe. Jugular vein blood samples were collected in heparinised glass vacuum tubes, cooled to 4 °C and transported

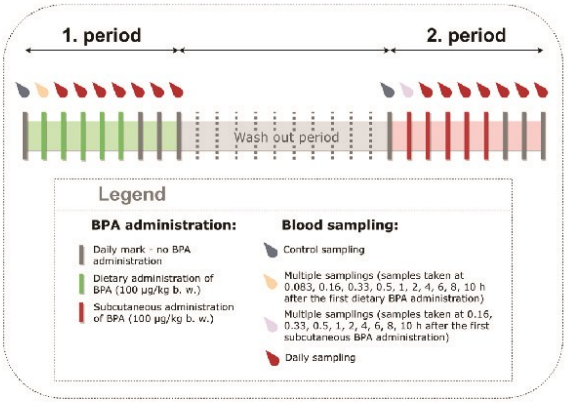

Figure 1. Study design with two experimental periods, BPA administration and blood sampling schedule for the ewe.

to the laboratory where blood plasma was separated by centrifugation at 2640 × g for 15 min. The plasma was transferred and stored in polypropylene (PP) tubes. Plasma samples were kept frozen at −20 °C until analysis.

A diagram illustrating the design of the study, including the two experimental periods, BPA administration and blood sampling schedule of the ewe, is provided below (Fig. 1).

Milk sampling was done in both periods. The ewe was hand-milked. On the first day of the experiment milk was collected six and 10 hours after the first BPA administration and every next day just before the following administration. Before the first administration in each period the ewe was milked and then the lamb was separated from the ewe during next six hours to allow estimation of the amount of BPA excreted in milk. The sampling period continued from the 5th until the 8th day of both periods, when there was no BPA administration. Milk was collected in PP containers and stored at −20 °C until analysis.

Blind samples of blood plasma from the sheep and suckling lamb and milk from the sheep were taken just before the start of both periods, to provide a baseline for the analysis.

The precautions taken to avoid contamination with BPA during sampling were: using glass vacuum tubes for blood collection and PP containers for milk collection.

Free BPA and total BPA sample analysis. BPA stock solution of 200 µg/mL was prepared in acetonitrile, while the intermediate and working standard solutions ranging from 2,000 to 1.0 ng/mL were further prepared in a mixture of acetonitrile and water at a ratio of 35: 65 (v/v). Working standard solutions ranging from 50,000 to 50 ng/mL for fortification of the total BPA samples were prepared in water with a small portion (≤20%, v/v) of ethanol or acetonitrile. All solutions were prepared using high purity deionised water obtained using a PureLab Option and PureLab Classic water purification system (Elga, Woodridge, Illinois, USA). The acetonitrile and methanol used were of HPLC gradient grade purity and purchased from J.T. Baker (Center Valley, PA, USA). Only high quality glass or labware were used for the sample analysis to avoid contamination with BPA during analysis.

Samples of the sheep blood plasma and milk were tested for the presence of both free (unconjugated) and total BPA (a sum of free and conjugated, mostly in a form of BPA-GLUC), of which the latter was determined indirectly by enzymatic conversion of the BPA-GLUC to free BPA. Sample aliquots of 1.5 and 5 mL were taken for the analysis of free BPA in the blood plasma and milk, respectively, while aliquots of 1.0 and 2.5 mL were taken to determine the total BPA and were diluted by 1.1 M Na-acetate buffer solution with pH values of 5.3 and 5.1 and volumes of 1.0 and 2.5 mL for the blood plasma and milk, respectively. Forty and 70 µL of ß-glucuronidase from *Helix pomatia*, type HP-2, ≥100,000 units/mL including also ≤7,500 sulfatase units/mL (Merck, Sigma-Aldrich, Darmstadt, Germany) were added to each sample of the blood plasma and milk, respectively. Samples were then incubated in a shaking water bath at 37 °C for 4 h.

The blood plasma and milk samples were further extracted by 6 and 10 mL of acetonitrile, respectively and ultrasonicated before being evaporated to dryness at 40–42 °C under a stream of N_2 using an N-evap 111 evaporator (Organomation Associates, Berlin, MA, USA). A further clean-up procedure included solid phase extraction (SPE) by the use of molecularly imprinted polymer (MIP) columns AFFINIMIP SPE Bisphenols, 6 mL, 100 mg (AFFINISEP, Petit-Couronne, France), while the additional use of a Chromabond HR-X phase, with 6 mL columns, 200 mg, and 85 µm particle size (Macherey-Nagel, Düren, Germany) was previously utilised for all deconjugated sample extracts, as described by Deceuninck *et al.*[17]. Final SPE extracts were re-dissolved in acetonitrile/H_2O (35/65, v/v) as follows: both free BPA blood plasma and milk samples in 0.5 mL, and total BPA blood plasma and milk samples in 1.0 and 0.5 mL, respectively. Fifty µL of the final extract were taken for the HPLC analysis.

HPLC measurements were performed using a Varian ProStar HPLC system (Varian Analytical Instruments, Walnut Creek, CA, USA), comprised of a tertiary pump (240 model), automatic injector (410 model), fluorescence detector (363 model), degasser and Galaxie 1.7.4.5 analytical software. Chromatographic separation was performed at room temperature by the gradient binary pumping of water and acetonitrile at a flow rate of 1 mL/min through a Hypersil Gold C18 analytical column, 150 ×4.6 mm, with a particle size of 3 µm, which was

protected with Hypersil GOLD 3 µ Drop in the guards (Thermo Scientific, Waltham, MA, USA). The mobile phase gradient was as follows: 0–2 min, 35% (v/v) of acetonitrile, gradient to 12 min, 35–50% (v/v) of acetonitrile, held to 20 min, gradient to 20.5 min, 50–35% (v/v) of acetonitrile, held to 21 min. The excitation and emission wavelengths of the fluorescence spectrophotometry analysis were set at 230 and 315 nm, respectively[18]. The results were evaluated in accordance with the external standard method using a standard calibration curve as a function of chromatographic peak areas and standard concentrations. Each sample series consisted of a matrix sample, obtained before the first periodic BPA administration (a baseline sample), five to seven animal study samples in duplicate and two baseline matrix samples fortified with BPA to control the recovery rate. The measured sample concentrations were corrected for the possible baseline matrix response and for the mean recovery of the respective series and then used as final results.

Validation of the analytical methodology used was performed to demonstrate its fitness for the stated purpose. Linearity was determined by the least-squares method to calculate regression and correlation parameters for six to seven standard concentration points per calibration curve (range 1.0–100 ng/mL), and for both matrices as a correlation between measured and added concentrations (ranges 0.25–10 µg/L and 1.0–50 µg/L for free and total BPA in blood plasma, respectively, 0.5–15 µg/L for both free and total BPA in milk). Mean recovery was evaluated by analysis of four to six fortified blank materials at two concentration levels at separate time points (blood plasma: free BPA 2 and 10 µg/L, total BPA 25 and 50 µg/L; milk: free BPA 2 and 5 µg/L, total BPA 5 and 10 µg/L). The within-laboratory reproducibility of the method was evaluated as the coefficient of variation (CV) of the determined and recovery values. The limit of detection (LOD) value was estimated as the BPA concentration in the retention time window where the analyte was to be expected, which corresponded to 3 × noise and was corrected for the blank matrix response.

Toxicokinetic analysis. Each entity (free BPA, BPA-GLUC, and total BPA) plasma concentration time course until the second BPA administration was first analysed using a noncompartmental approach to obtain the estimates of the area under the concentration–time curve extrapolated to infinity (AUC), maximum concentration in plasma and time when it occurs (c_{max} and t_{max}, respectively). AUC was calculated using the linear trapezoidal method and extrapolated to infinity by addition of the term C_{last}/λ_z, where C_{last} is the last quantified concentration measurement and λ_z is the terminal slope of the concentration profile in the semi-log plot calculated by linear regression. t_{max} and c_{max} were reported as observed. AUC values were used to estimate clearance (CL) as $CL = Dose/AUC_{sc}$ and relative bioavailability after dietary administration (F_r) as $F_r = AUC_{po}/AUC_{sc}$. The indexes po and sc refer to the route of administration (dietary and subcutaneous, respectively) and Dose is the single BPA dose (100 µg/kg b. w.). Note that CL can be estimated only after intravenous administration. Our estimate of CL is therefore apparent clearance, i.e. assuming complete bioavailability after subcutaneous administration.

Subsequently, all TK data after both routes of administration were simultaneously fitted to a one- and two-compartment model with first-order absorption and elimination. The estimated parameters were CL, volume of the central and peripheral compartment (Vc and Vp, respectively), distribution clearance (Q), absorption rate constants after subcutaneous and dietary administration ($k_{a\,sc}$ and $k_{a\,po}$, respectively) and relative bioavailability (F_r). Parameter fitting was performed using ADAPT II software[19] with the maximum likelihood method and a proportional variance model, $V_i = (\sigma \times Y_i)^2$, where V_i is the variance of the i-th data point and Y_i is the value predicted by the model. The Akaike Information Criterion (AIC) value was used to select the model.

Permeation of free BPA, BPA-GLUC and total BPA into milk was modelled as a first order process $dA_m/dt = k_m \times Cp(t)$, where dA_m/dt is the transfer rate in µg/h, $C_p(t)$ is the BPA plasma concentration at time t, and k_m is the transfer rate constant. k_m was estimated by simultaneous fitting of the amounts excreted into milk up to six hours after the first subcutaneous and dietary administration, with TK parameters for the plasma data fixed to previously estimated values. The amounts excreted in milk up to 6 h were approximated by multiplication of the concentration in milk at 6 h by 0.25 L, i.e. assuming an average milk yield of 1 L/day. We tested the hypothesis that only free BPA is transferred into milk and subsequently conjugated in the mammary gland, i.e. fixing the TK parameters to the values estimated for the free BPA versus the hypothesis that BPA-GLUC is also transferred, i.e. fixing the TK parameters to the values obtained for the conjugated and total BPA.

Results

Validation of the analytical methodology used. The validation parameters of the BPA blood plasma and milk analysis are presented in Table 1. The method was linear for BPA standards and matrices, as proved by the determination coefficients (r^2) of ≥0.999 and ≥0.991, respectively. Mean recoveries for free and total BPA in the blood plasma were 82.3 and 49.5%, respectively and in the milk 62.9 and 54.3%, respectively. The total BPA refers to the sum of free and BPA-GLUC. The CVs of the concentrations detected and recovery in the fortified samples were from 1.5–24.4% under within-laboratory reproducibility conditions. The LOD values were 0.05–0.1 µg/L and 0.2–0.4 µg/L for the free and total BPA determination, respectively, and differed according to a more comprehensive chromatographic background in the total BPA extracts.

TK analysis. BPA levels were checked before conducting both the first and second part of the experiment to provide a baseline for the analysis. The ewe entered the first part of the experiment with 0.05 and <0.4 µg/L of the free and total BPA in the blood plasma, respectively, and with <0.1 and 0.31 µg/L of the free and total BPA in milk, respectively. Just before the start of the subcutaneous administration, the ewe's blood plasma contained 0.15 and 0.72 µg/L of the free and total BPA, respectively, while its milk contained <0.1 and 0.35 µg/L of the free and total BPA, respectively.

Parameter		Free BPA				Total BPA			
		Blood plasma		Milk		Blood plasma		Milk	
Linearity									
Standards	Range (ng/mL)	1.0–100							
	Correlation (r^2)	0.9993–0.9999							
Matrix	Range (µg/L)	0.25–10		0.5–15		1.0–50		0.5–-15	
	Correlation (r^2)	0.9956		0.9984		0.9982		0.9908	
Recovery and precision	Added concentration (µg/L)	2	10	2	5	25	50	5	10
	Recovery (s.d.) (%)	76.13 (2.26)	88.37 (1.29)	56.76 (13.83)	69.08 (11.90)	57.11 (13.64)	41.96 (2.99)	57.67 (8.53)	51.04 (10.61)
	CV (%)	2.97	1.46	24.36	17.23	23.88	7.13	14.79	20.78
LOD (µg/L)		0.05		0.1		0.4		0.2	

Table 1. Validation results of BPA determination in blood plasma and milk.

Parameter		Free BPA	BPA-conjugate	Total BPA
Subcutaneous administration	CL (L/h/kg)	3.0055	0.3650	0.3258
Dietary administration	F_r (%)	3.8	149	134

Table 2. CL = clearance, F_r = relative bioavailability, dietary vs. subcutaneous administration. TK parameters of the noncompartmental TK analysis following the first dietary and subcutaneous BPA administration.

Parameter	Free BPA	BPA-GLUC	Total BPA
CL (L/h/kg)	3.12 (7.2%)	0.388 (9.9%)	0.343 (9.1%)
V_c (L/kg)	2.45 (23.4%)	2.17 (13.3%)	1.89 (12.4%)
$k_{a\,sc}$ (h^{-1})	0.455 (16.1%)	2.57 (29.1%)	2.59 (27.1%)
Q (L/h/kg)	0.425 (39.4%)	/	/
V_P (L/kg)	5.75 (27.4%)	/	/
$k_{a\,po}$ (h^{-1})	6.39 (67.1%)	3.24 (19.2%)	3.44 (20.0%)
Fr (%)	4.52 (29.9%)	115 (12.9%)	101 (12.4%)

Table 3. CL = clearance, V_c = volume of central compartment, $k_{a\,sc}$ = absorption rate constant after subcutaneous administration, Q = distribution clearance, V_p = volume of peripheral compartment, $k_{a\,po}$ = absorption rate constant after dietary administration, F_r = relative bioavailability, dietary vs. subcutaneous administration, RSE = relative standard error, calculated by dividing standard error by mean value (%). TK parameters of the BPA (RSE%) following repeated dietary and subcutaneous administration.

Comparison of the plasma concentration-time profiles. The maximum plasma concentration of free BPA obtained after subcutaneous administration was higher than after dietary administration. In addition, free BPA exposure was prolonged after subcutaneous administration compared to the dietary route of intake. With the dietary route, c_{max} of free BPA in plasma was 2.15 µg/L and was obtained very quickly, at 0.33 h. For the subcutaneous route, c_{max} of free BPA was 6.41 µg/L and was obtained after 2 h.

The c_{max} values of BPA-GLUC were similar for both routes of exposure and were 49.64 µg/L (t_{max} = 1 h) for subcutaneous administration and 41.3 µg/L (t_{max} = 0.33 h) for dietary administration.

The same seems to be valid for the c_{max} of total BPA, where c_{max} after subcutaneous administration was 55.6 µg/L (t_{max} = 1 h) and c_{max} after dietary administration was 43.46 µg/L (t_{max} = 0.33 h).

The AUC of free BPA was 33.3 µg h/L for subcutaneous administration and 1.28 µg h/L for dietary administration. However, for BPA-GLUC and total BPA the AUC was 274 µg h/L and 307 µg h/L for subcutaneous administration and 409 µg h/L and 410 µg h/L for dietary administration, respectively.

Administration by the subcutaneous route led to a higher overall internal exposure to free BPA and lower internal exposure to BPA-GLUC/total BPA compared to the dietary route.

Clearance and relative bioavailability of free BPA, BPA-GLUC and total BPA obtained with noncompartmental TK analysis are presented in Table 2.

The TK parameters obtained with the TK model of the free BPA, BPA-GLUC and total BPA are gathered in Table 3. Blood plasma BPA-GLUC and total BPA concentration time courses were described with a one-compartment model, while a two-compartment model was more suitable for free BPA.

Figure 2 shows the plasma free BPA, BPA-GLUC and total BPA concentration-time profiles after repeated dietary and subcutaneous exposure of the ewe during the five days of a daily BPA administration of 100 µg/kg b. w. and the subsequent three days of no BPA administration. The concentration-time profiles between the two routes varied slightly for BPA-GLUC and total BPA, and markedly for the free BPA.

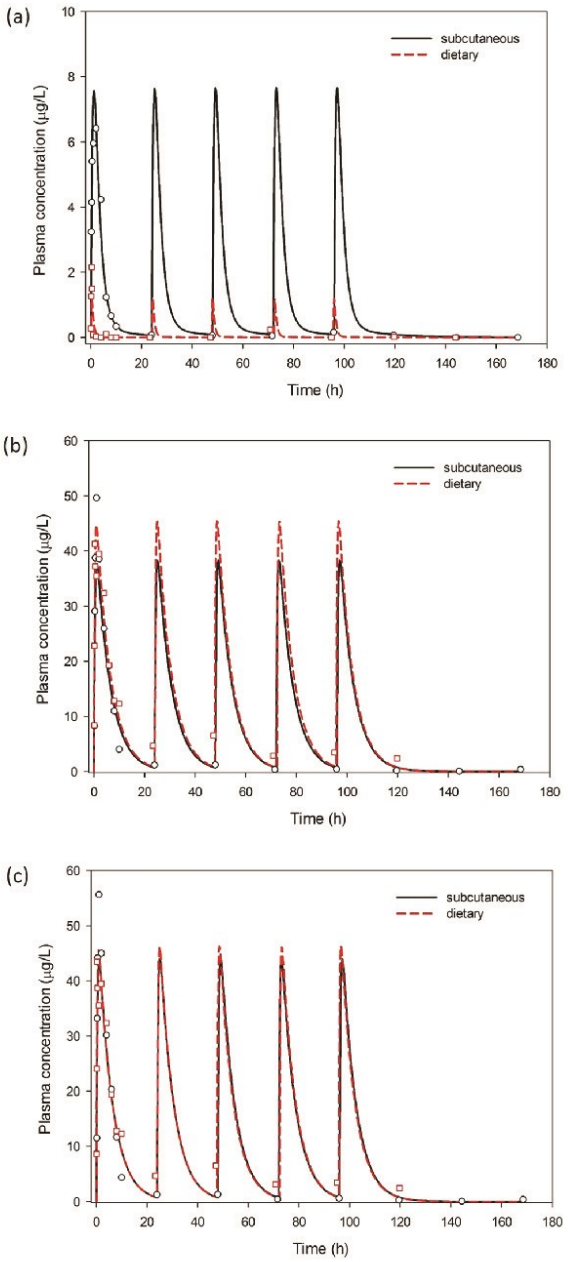

Figure 2. Time course of free BPA (**a**), BPA-GLUC (**b**) and total BPA (**c**) plasma concentration.

The ewe was administered 100 µg/kg b. w./day of BPA sc or po for five days, one time per day in the morning. The administration was stopped the sixth day of the experiment, while the sampling continued until the eighth day. Blood samples were collected at 0.08 – 0.17 – 0.33 – 0.5 – 1 – 2 – 4 – 6 – 8 – 10 – 23.33 – 46.58 – 69.35 – 92.35 – 116.1 – 140.1 – 164.1 hours after the first dietary administration and 0.17 – 0.33 – 0.5 – 1 – 2 – 4 – 6 – 8 – 10 – 23.92 – 47.84 – 71.34 – 95.17 – 118.67 – 143 – 167.5 hours after the first subcutaneous administration.

Estimation of the free BPA, BPA-GLUC and total BPA elimination into the milk. Given our model assuming passive transfer (first-order) of BPA into milk, it is more likely that only free BPA is transferred into the mammary gland (AIC = −5.066 for the BPA-GLUC and AIC = −5.031 for the total BPA), versus the hypothesis that BPA-GLUC is also transported (AIC = −3.900 for the BPA-GLUC and AIC = −2.254 for the total

BPA). The estimated rate constants of transfer into milk (estimate (RSE%)) were 0.00832 L/h (0.1%) for the free BPA, 0.01839 L/h (3.6%) for the BPA-GLUC and 0.02669 L/h (2.4%) for the total BPA.

For the first part of the experiment (dietary administration), the percentage of the dose eliminated with milk was 0.0002% (RSE = 0.1%) for the free BPA, 0.00045% (RSE = 3.6%) for BPA-GLUC and 0.00066% (RSE = 24%) for total BPA.

For the second part of the experiment (subcutaneous administration), the percentage of the dose eliminated with milk was 0.00453% (RSE = 0.1%) for the free BPA, 0.01001% (RSE = 3.6%) for BPA-GLUC and 0.01452% (RSE = 2.4%) for total BPA.

Regarding the suckling lamb, which drank milk after its mother was administered with BPA by the dietary or subcutaneous routes, there were only traces of BPA in the samples of its plasma.

Discussion

The purpose of this study was to investigate the TK of BPA and to evaluate its elimination into the sheep milk after two different routes (po and sc) of repeated low dose BPA administration.

A comparison of the plasma concentration-time profile for the basic TK parameters of the two administration routes was made using the noncompartmental approach. Regarding the comparison of both routes of BPA administration, our preliminary findings are similar to those of Guignard *et al.*[20], where the TK parameters for the same routes of administrations but with higher dose regimens were compared. The formulations for the dietary and as well for subcutaneous route of administration were similar in both studies. In our study, the c_{max} of free BPA for dietary administration was obtained quickly (0.33 h). In their study, mean c_{max} was attained 0.12 h for two ewes and 0.20 h for two others. For the subcutaneous route, c_{max} in our study was obtained after 2 h, in their study it was obtained after 2 h for three ewes and after 1 h for one ewe. In our study, the free BPA c_{max} for the subcutaneous route was three-fold higher than for the dietary route and in their work the free BPA c_{max} for the subcutaneous route was 4.6 ± 1.5-fold higher than for the dietary route. Our study demonstrates a higher cumulative (AUC) internal exposure to free BPA after subcutaneous administration compared to the dietary route, which is in line with the findings of Guignard *et al.*[20]. In their study, the relative bioavailability of BPA for the dietary as compared to subcutaneous route was 3.3 ± 0.3%. In our work, the relative bioavailability of BPA for the dietary as compared to subcutaneous route was 4.5%. Both this earlier work and the current study were also in agreement with regard to the BPA-GLUC concentration time course. Unlike free BPA, the BPA-GLUC concentration time courses are very similar for the two routes of exposure. Although our preliminary study was made with only one animal, the acquired data are in agreement with those of Guignard *et al.*[20]. This is important, as measured concentrations from our study were the base for the TK model, which we used to evaluate the elimination of BPA into the sheep milk. Sampling of the milk was possible only at a couple of sampling points, and thus it was not possible to make time-concentration profiles for it. However, the measurments of BPA and BPA-GLUC in blood plasma and our TK model enabled us to estimate the percentage of the dose eliminated with milk, which was less than 0.1% for free BPA, BPA-GLUC and total BPA, regardless of the route of administration. This result is comparable with the results of Snyder *et al.*, where they found only a small fraction of the ^{14}C labelled BPA (0.63 +/− 0.13 µg/equiv/mL) 8 hours after dosing[10]. Regarding free BPA, BPA-GLUC and total BPA, it is already indirectly proven in rats that free BPA is transferred into the mammary gland to a greater extent than BPA-GLUC[13,21]. Given our TK models, the same was true in our study for the ewe. In the first model we were assuming passive transfer (first-order) of free BPA into milk, and in the second we were assuming that BPA-GLUC would also be transported. Based on the lower value of the Akaike information criterion (AIC), which is one of the indexes, which are showing model's goddness of fit, in the first model, it is more likely that only free BPA is transferred into the mammary gland. Nevertheless, it was reported that the major molecular species in the milk of rats after oral administration of ^{14}C-BPA was BPA-GLUC. The concentrations measured in milk six hours after BPA administration (dietary and subcutaneous) in our study show the same result. Six hours after dietary administration, the concentration of free BPA was 0.05 µg/L and the concentration of BPA-GLUC was 0.78 µg/L. Similarly, the concentrations of free BPA and BPA-GLUC after subcutaneous administration were 0.87 and 1.89 µg/L, respectively. Regarding the BPA-GLUC in the milk, we hypothesise that free BPA is passively transferred into the mammary gland, and subsequently conjugated in its glucuronidated form presumably by the uridine 5′-diphospho-glucuronosyltransferase (UDP-glucuronosyltransferases) in the mammary gland. Only a few studies have evaluated UDP-glucuronosyltransferases (UGTs) presence in breast tissue. Expression of UGT2B10, UGT2B11, UGT2B15 and UGT2B UGT1A10 and UGT2B7, and UGT2B11 enzymes have been proven in humans, and the results of Street *et al.* confirm the capability of glucuronidation of BPA in human breast tissue, although with glucuronidation activities that are much lower (by more than 100,000-fold) compared with those seen in the liver[22]. There are currently no (to the best of our knowledge) known studies that have evaluated the presence of UDP-glucuronosyltransferases in the ewe mammary gland, although it seems reasonable to assume that the mammary glands of all mammals are equipped with comparable detoxifying mechanisms.

The above mentioned concentrations of free BPA measured in this study are well within the range with the concentrations found in raw milk measured in a recent Italian monitoring study, where the concentrations of free BPA ranged from 0.081–2.492 µg/L[23]. However, the concentrations measured in commercial milk samples were generally higher (from 14.0 to 521.0 µg/L)[6], meaning that the BPA load in consumption milk is greater at the end of the production line and during further processing.

We report that three years after the end of the experiment, the ewe and the lamb are in a good health condition. There were no reproductive abnormalities reported in the ewe in the past three years. After the experiment, the ewe had offspring each year. In the year 2018, the offspring were two healthy male lambs, while in the year 2019, the offspring were one healthy male and one healthy female lamb. The grown-up lamb developed normally, has been recently mated for the first time and is currently pregnant (12. 3. 2020). Both animals have appropriate body weight and there is no known history of disease.

Conclusion

In this preliminary study, we are presenting the detected BPA and BPA-GLUC concentrations in the milk samples obtained from the ewe administered by two different routes of administration. With the TK model, on the basis of blood plasma and milk measurements, we were able to estimate the percentage of the eliminated BPA in the milk, which is minimal regardless of the administered route of BPA. In addition, with our TK model we can suggest that in sheep, being a large animal model, BPA is transferred in the milk in the mammary gland mostly in its free form. As BPA-GLUC concentrations were higher than free BPA concentrations in milk, and that with regards to our TK model, only BPA can cross the barrier between blood and mammary gland, we are estimating that free BPA is probably subsequently metabolised in mammary gland. Further studies are needed to confirm preliminary findings in this study, especially in a view of greater number of animals, different phases of lactation and different BPA doses.

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Author contributions

M.P., V.C.F., A.Š. and S.Š. conceived the study, participated in the design and coordination. S.Š. and V.C.F. performed the analysis of the samples. I.G. and S.Š. performed the toxicokinetic analysis. S.Š., V.C.F. and I.G. participated in the writing of the manuscript. Figure 1 was created by S.Š. and Fig. 2 was created by I.G. All authors have read and approved the final manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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2.2 DETERMINACIJA PROSTEGA IN CELOKUPNEGA BISFENOLA A V URINU IN IZTREBKIH OVCE Z METODO HPLC S FLUORESCENČNO DETEKCIJO

Determination of free and total bisphenol A in the urine and feces of orally and subcutaneously dosed sheep by high-performance liquid chromatography with fluorescence detection

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Izvleček

V raziskavi smo razvili analizno metodo, ki je omogočila preučevanje izločanja prostega BPA, celokupnega BPA in njegovega glavnega metabolita BPAG. Vzorce urina in iztrebkov smo pridobili iz postopka, v katerem smo eni ovci pasme istrska pramenka pet dni zaporedoma vnašali 100 µg/kg BPA. Vsebnost prostega BPA in celokupnega BPA v vzorcih smo izmerili s tekočinsko kromatografijo visoke ločljivosti (HPLC) na obrnjeni stacionarni fazi ter s fluorescenčno detekcijo. Zaradi dobrega izkoristka, natančnosti in občutljivosti sta se metodi izkazali za uporabni tudi za nadaljnje ekotoksikološke raziskave prostega BPA, BPAG in celokupnega BPA. Rezultate smo primerjali z vrednostmi, ki so jih raziskovalci ugotovili v živalskih izločkih na farmah.

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Determination of free and total bisphenol A in the urine and feces of orally and subcutaneously dosed sheep by high-performance liquid chromatography with fluorescence detection

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ABSTRACT

An analytical procedure has been introduced to enable a study of the excretion of free bisphenol A (BPA), total BPA and its main metabolite bisphenol A glucuronide (BPA–GLUC). In the experiment, in which 100 μg/kg b. w. BPA was administered daily to one Istrian Pramenka sheep for 5 days with consecutive urine and feces samples being taken, BPA and total BPA were determined in samples using high-performance liquid chromatography (HPLC) with fluorescence detection. Because of their good recovery, precision, and sensitivity, the methods have also proved applicable to further ecotoxicological studies of free BPA, BPA–GLUC and total BPA. The results were subsequently compared with reported field studies of BPA in livestock excreta.

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Introduction

Bisphenol A (BPA) (Table 1), one of the most widely produced and used chemicals in the world, is a well-known xenobiotic present in numerous daily products, mainly in plastics, particularly in polycarbonate resins and epoxy resins.[1]

Recognized as an endocrine disruptor, BPA is capable of altering the endocrine function by imitating or blocking the endogenous hormones.[2] Studies regarding its impact were mainly directed toward vertebrate species[1] or aquatic invertebrate species.[3] In recent years, some studies were also directed toward the impact of BPA on the edaphic environment and soil life. These studies were particularly conducted on isopods[4,5] and earthworms,[6–9] due to the detected presence of BPA in agricultural soils.[3]

BPA can reach soil life through sewage sludge or biosolids that are used to fertilize farmland.[10] However, little data is available about BPA in farm animal excreta (Table 2),[11–16] even though livestock excreta are still directly applied to the soil in some farming practices. In addition, the different policies of various countries allow the producers of animal feed to include waste food, still packaged, in animal feed. It is thus highly likely that BPA could enter animal feed and could be subsequently eliminated with animal excreta. As such, animal excreta would be, together with biosolids, responsible for the BPA contamination of the agricultural soil. Yet, information on the occurrence of BPA in animal feed for livestock is lacking.

The majority of studies in which the excretion of BPA is evaluated, are toxicokinetic studies. Most of these determine BPA excretion in the urine of different animal models (sheep, rats, monkeys, pigs), and some even report fecal excretion (rats, monkeys).[17–20]

There are a few published analytical methods for the determination of bisphenols in animal urine and feces, and those that do exist are principally derived[21] from the methods used in human occurrence and exposure studies.[22] A combination of scintillation counting and a radio-high performance liquid chromatography (HPLC) was used for determination of both free and total (free + conjugated) ^{14}C-BPA in experimentally dosed monkeys[19] and rats.[18] Two liquid chromatography—tandem mass spectrometric (LC-MS/MS) methods in Twaddle et al.[23] and Yang et al.[24] determined total deuterated BPA (d6-BPA) and bisphenol AF (BPAF) in both the urine and feces of experimentally dosed rats, respectively, while an LC-MS/MS method of Zhang et al.[11,12] determined free (aglycone) BPA in the feces obtained in field studies of various livestock animals. With regard to the differences in these earlier methods that all utilized enzymatic hydrolysis by ß-glucuronidase for the deconjugation step, Lacroix et al.[17] pioneered the simultaneous quantification of BPA and BPA–GLUC in sheep's urine by LC-MS/MS.

The objective of our work was to introduce a sensitive and selective analytical method for determination of BPA and total BPA in urine and feces after consequent dietary and subcutaneous administration of 100 μg/kg b. w. of BPA

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Table 1. Chemical names, CAS numbers, synonyms, and chemical structures of the bisphenol A (BPA) and BPA–glucuronide (BPA–GLUC).

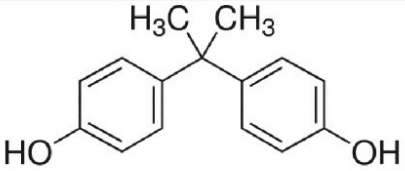
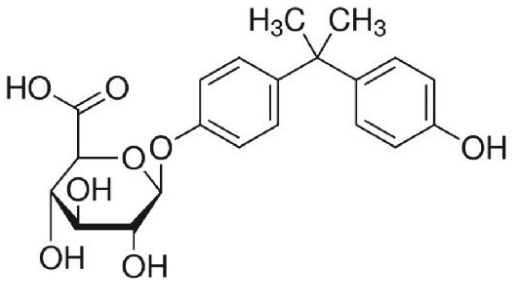
Chemical, abbreviation	CAS number	Synonym	Chemical structure
Bisphenol A, BPA	80-05-7	2,2-bis(4-hydroxyphenyl)propane, 4,4′-isopropylidenediphenol	
Bisphenol A mono-β-D-glucuronide, BPA–GLUC	267244-08-6	4-[1-(4-hydroxyphenyl)-1-methylethyl]phenyl β-D-glucopyranosiduronic acid	

Table 2. The determined (free) BPA concentrations in livestock excreta from the available field studies.

Sample	Animal species	Free BPA concentration	Reference
Feces (μg/kg)	Milking cow	4.1–10.9	[11]
	Replacement cow	7.4–10.8	
	Piglet	ND	
	Barrow	ND–6.0	
	Sow	ND	
	Broiler (female)	11.8–12.0	
	Broiler (male)	0.9–13.0	
	Laying hen	5.4–9.8	
	Brood hen	ND	
Urine (μg/L)	Replacement cow	0.23–0.31	
	Barrow	0.42–0.45	
	Sow	0.22–0.26	
Fresh feces (μg/kg)	Milking cow	2.3–2.7	[12]
	Beef cattle (bull)	3.3–4.1	
	Sow	ND	
	Broiler chicken	ND	
Fresh urine (μg/L)	*Sheep**	*1.0–1.6 ng/L*	
	Milking cow	0.35–0.41	
	Beef cattle	1.95–2.12	
	Sow	0.35–0.41	
Manure (μg/kg)	Poultry	up to 207	[13]
Manure (μg/kg)	Hen	ND–166.5	[15]
	Duck	ND–178.9	
	Swine	ND–361.8	
	Cow	ND–33.3	
Manure (μg/kg)	Swine	ND	[16]
Liquid manure (μg/kg d. w.)	Pig and cow-fattening facilities	61.1–1,112	[14]

ND = not detected
*There is dispute in the article regarding the BPA concentrations in sheep, whether the concentration was measured in urine or in feces.

to a sheep, and compare the obtained concentrations of BPA, total BPA and of the main metabolite bisphenol A glucuronide (BPA–GLUC) (Table 1) with those in the reported field studies in the literature.

Materials and methods

Experimental design

Chemicals

Bisphenol A of ≥ 99% purity (Merck, Sigma-Aldrich, Darmstadt, Germany) was dissolved in absolute ethanol for the dietary route and in corn oil for the subcutaneous route of administration. The dosage administered to the sheep was adjusted to the body weight recorded on the day of the administration. For dietary administration BPA solution in absolute ethanol was spilled onto the pellet ration (on average 1.18 mL of solution on 50 g of pellets), and applied on the morning dosage of pellets (400 g). For subcutaneous administration, injection of BPA solution was performed in the shoulder area (2.9 mL). BPA solutions were stored in sealed amber glass bottles at the ambient temperature for the entire experiment. Solution preparation, sample

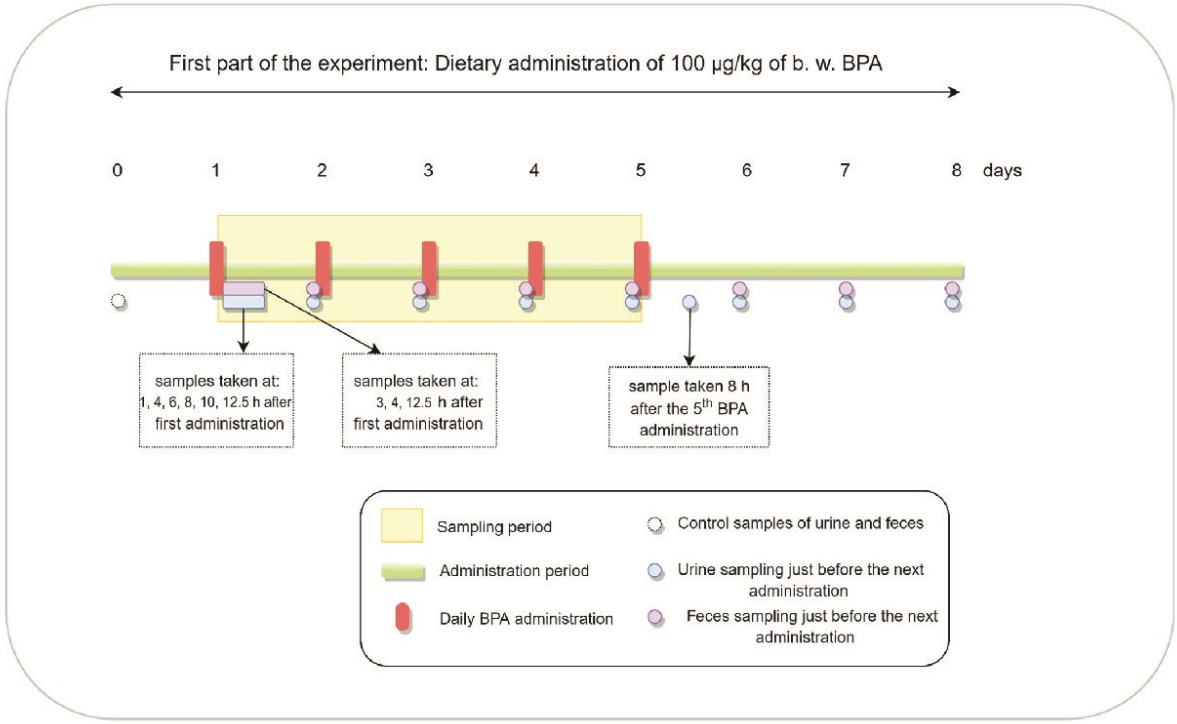


Figure 1. Study design with dietary experimental period, BPA administration (100 µg/kg b. w. per day), urine and feces sampling scheme for the ewe.

processing and assays were performed with the materials either made of glass or of BPA-free plastics.

Sampling regime

All animal procedures were carried out in accordance with ethical standards and approved by the Administration of the Republic of Slovenia for Food Safety, Veterinary Sector and Plant Protection with permission no. U34401-3/2015/8.

The study was performed on one stabled, healthy, lactating Istrian Pramenka sheep in a sheepfold at the Infrastructure Center for Sustainable Recultivation at Vremščica belonging to the Veterinary Faculty of the University of Ljubljana, Slovenia. The 6-year-old ewe weighed 59 kg. It was clinically healthy, as indicated by medical (temperature, breathing and rumination frequency, pulse rate), haematological and biochemical examination. It was penned individually and kept under natural temperature and photoperiodic conditions, with free access to water, hay and salt. The sheep was fed twice a day with 400 g plant-based pellets (SchafKorn Lac, Unser Lagerhaus Warenhandels Ges., Austria). Eventual contamination of the experimental environment was checked by testing of drinking water and pellets by HPLC analysis prior to the experiment. The analysis revealed the slight presence of BPA of 0.02 µg/L in drinking water and 5 µg/kg in pellets.

In the first period, the ewe received BPA in its diet (100 µg/kg b. w.) for five consecutive days. The ewe ingested all pellets within 2–9 minutes. During the second period, after a 13-day wash-out period, the same ewe was injected in the shoulder area with 100 µg/kg b. w. of BPA subcutaneously per day for five consecutive days. The sampling scheme for urine and feces after dietary administration of BPA is shown in Fig. 1.

The experimental design was the same for the part of the experiment with subcutaneous administration of BPA, with only small differences in some sampling times for the feces samples, as follows. On the first day of the experiment there were different time periods of feces collection, which were 4.5, 5, 6.15 and 8 hours after the first subcutaneous administration of BPA, and on the second day, sampling was done 12 hours after the administration of BPA.

Urine samples were collected with the stimulation of sheep to urinate. They were collected in a clean glass and stored in a laboratory screw cap bottle. Feces samples were collected fresh from the barn floor, right after defecation. The samples were stored in polypropylene (PP) tubes. Immediately after the sampling, urine and feces samples were frozen at −20 °C. The samples were kept frozen until analysis.

For study sample concentration measurements, the samples from the ewe, taken just before the start of the experiment were used as baseline samples. Additional urine and feces samples from multiple other stabled sheep from the same herd of the same breed, nutrition and physiological status, were also taken simultaneously to enable enough biological material for validation series. These sheep were not treated with BPA and were dislocated from the ewe with a lamb included in the experiment.

Analytical method

Reference standard materials

The certified reference standard of BPA was obtained as a powder of 99.0% analytical purity from Sigma-Aldrich (Merck, Darmstadt, Germany). The reference standard solutions were prepared in high-quality dark brown glassware. The stock standard solution was prepared at a concentration of 200 μg/mL in acetonitrile (MeCN) and kept frozen (at −20 °C). Working standard solutions for the calibration curve and fortification of the samples determining free BPA were prepared in 35% (v/v) MeCN in H_2O. At concentrations of ≥ 50 ng/mL they were kept refrigerated (at 4–8 °C), whereas at concentrations below 50 ng/mL they were prepared on a daily basis. The working standard solutions used for fortification of the samples determining total BPA, were prepared by dissolution of a reference powder of BPA in H_2O or by proper dilution of a stock standard solution (in MeCN) with H_2O. At concentrations from 10,000–100,000 ng/mL they were kept frozen (at −20 °C), whereas at concentrations ≤ 500 ng/mL they were prepared on a daily basis.

Reagents and consumables

The high-purity deionized water used with resistivity of 18.2 MΩ.cm was obtained using a PureLab Option and PureLab Classic water purification system (Elga, Woodridge, Illinois, USA). The MeCN and methanol (MeOH) used, which were HPLC gradient-grade purity, were supplied by J.T. Baker (Center Valley, PA, USA). The aqueous solution of enzyme ß-glucuronidase from *Helix pomatia* Type HP − 2, with ≥100,000 U/mL (and ≤7,500 U/mL of sulfatase activity), the sodium acetate anhydrous for analysis, acetic acid (glacial) 100% anhydrous for analysis and formic acid 98–100% for analysis, were supplied by Merck (Darmstadt. Germany). Sodium acetate buffer of 1.1 M and with pH values of 4.8 and 4.9 was prepared by mixing of aqueous 1.1 M sodium acetate and 1.1 M acetic acid (glacial) in a ratio of 59:41 (v/v) and 64.5:35.5 (v/v), respectively.

The solid-phase extraction (SPE) columns used were Chromabond HR-X, 6 mL, PP, with 85 μm particle size and 200 mg of sorbent, which were supplied by Macherey-Nagel (Düren, Germany), and molecularly imprinted polymer (MIP) AFFINIMIP® SPE Bisphenols, 6 mL, with 100 mg of sorbent, which were supplied by AFFINISEP (Petit-Couronne, France). The centrifuge tubes (15 mL, conical, screw cap, PP) were supplied by Isolab (Wertheim, Germany), and the centrifuge tubes (15 mL, conical, glass) were purchased from Brand (Wertheim, Germany). The dark glass, 1.5−mL vials for HPLC were purchased from La-Pha-Pack (Langerweche, Germany).

Equipment

The homogenization of the sheep's feces was performed using a Tube Mill control with a metal mixing chamber (Ika, Staufen, Nemčija). An electronic balance Vibra AJ − CE/AJH − CE (± 0.001 g), an incubator shaker Vibromix 403 RVI, a Vibromix 10 Vortex mixer and a centrifuge Centric 350 were obtained from Domel (Železniki, Slovenia). A Transsonic 460/H ultrasonic bath was acquired from Elma (Singen, Germany). An SPE vacuum manifold Visiprep with 24 flow control valves was supplied by Merck (Darmstadt, Germany), and an N-EVAP 111 evaporator was provided by Organomation Associates (Berlin, MA, USA). The HPLC system used was a Varian ProStar (Varian Analytical Instruments, Walnut Creek, CA, USA), which comprised a tertiary pump (240 model), an automatic injector (410 model), a fluorescence detector (363 model), a degasser and Galaxie 1.7.4.5 analytical software.

Sample extraction and clean-up

Samples of the sheep's urine and feces were tested for the presence of both free (aglycone) and total (a sum of free and conjugated) BPA. Conjugated BPA was determined by an enzymatic deconjugation of the glucuronide bond, followed by subtraction of the free BPA from the total BPA.

Regarding the sheep's urine, for the determination of the free BPA an aliquot of 1 ± 0.005 mL of the homogenized sample was transferred into a 15-mL plastic (PP) centrifuge tube and diluted by 4 mL of H_2O, while for the determination of the total BPA a sample aliquot of 0.5 ± 0.005 mL was diluted by 1.5 mL of 1.1 M sodium acetate buffer with pH 4.8, 20 μL of the ß-glucuronidase from *Helix pomatia* and incubated by shaking for 16 hours at 37°C. A further clean-up of both free and total BPA was performed by solid phase extraction (SPE) using two SPE sorbents, namely Chromabond HR − X and molecularly imprinted polymer (MIP) AFFINIMIP® SPE Bisphenols according to the procedure developed by Deceuninck et al.[25] with some modifications and omitting the derivatization step. In detail, urine samples were applied slowly under gravity (1 drop/5 sec) onto the Chromabond HR-X cartridge, pre-conditioned by 10 mL of H_2O and 10 mL of MeOH. The cartridge was then washed under gravity (1 drop/sec) with 6 mL of H_2O, 8 mL of MeOH/H_2O (10/90, v/v) and 4 mL of MeOH/H_2O (60/40, v/v), and was shortly sucked by vacuum at the end of washing step. The solid phase extract was eluted by 10 mL of MeOH under gravity (1 drop/2 sec) into a 15-mL glass tube and was evaporated under a N_2 stream at 40 °C just to dryness. The residue was re-dissolved in 0.2 mL of MeCN, ultrasonicated for 5 min, diluted by 5 mL of H_2O, vibromixed, and applied slowly under gravity (1 drop/5 sec) onto the AFFINIMIP® SPE Bisphenols cartridge, pre-conditioned by 10 mL of formic acid/MeOH (2/98, v/v, prepared on a daily basis), 4 mL of MeCN and 4 mL of H_2O. The cartridge was washed under gravity (1 drop/sec) with 5 mL of H_2O, 3 mL of MeCN/H_2O (40/60, v/v) and 3 mL of MeCN, and was sucked by vacuum for 2–3 min at the end of washing step. The solid phase extract was eluted by 4 mL of MeOH under gravity (1 drop/2 sec) into a 15 mL glass tube and was evaporated under a N_2 stream at 40 °C just to dryness.

Final free BPA extracts were re-dissolved in 0.8 mL of MeCN/H_2O (35/65, v/v), ultrasonicated for 2 min, and vibromixed, while total BPA extracts were re-dissolved in 1 mL of the same solution, ultrasonicated for 2 min,

vibromixed, and further diluted in a volume ratio of 1:24 or 1:4 (v/v), depending on the analyte concentration level. An aliquot of the final sample extract was transferred into a HPLC vial.

Regarding the sheep's feces, an aliquot of 0.5 ± 0.003 g of the homogenized moist sample was put into a 15−mL plastic (PP) centrifuge tube. For the determination of the free BPA, it was extracted by 8 mL of MeCN, by vigorous vibromixing for 2 min and ultrasonication for 13 min. After repeated vibromixing for 1 min, the sample was centrifuged at room temperature for 10 min at 2640×***g*** and re-extracted using 2 mL of MeCN. For the determination of the total BPA, a sample was diluted by 1.5 mL of 1.1 M sodium acetate buffer with pH 4.9, then 20 μL of the ß-glucuronidase from *Helix pomatia* was added, and the sample was incubated by shaking for 4 hours at 37°C. Total BPA was extracted from the buffered feces using 6 and 2 mL of MeCN in the same manner as free BPA. The combined MeCN supernatant for both free and total BPA residue was transferred into a 15−mL glass centrifuge tube and evaporated under a N_2 stream at 40°C to an aqueous residue. The free BPA sample extract was re-dissolved in 0.3 mL of MeOH, vibromixed, ultrasonicated and diluted by 4.7 mL of H_2O, while the total BPA sample extract was re-dissolved in 3 mL of MeOH/H_2O (10/90, v/v) solution. From this step onward the procedure basically followed the analytical method of Deceuninck et al.,[25] in the same manner as described for urine samples. Final sample extracts were re-dissolved in 0.5 mL of MeCN/H_2O (35/65, v/v), ultrasonicated for 5 min, vibromixed, centrifuged at room temperature for 10 min at 2640×***g***, and transferred into a HPLC vial.

HPLC analysis

A 50 μL aliquot of the final urine and feces sample extract was taken for the high-performance liquid chromatography (HPLC) analysis. A Hypersil GOLD C18 (150 × 4.6 mm, 3 μm particle size) analytical column was used which was protected by a Hypersil GOLD 3 μ drop in guard cartridges (Thermo Scientific, Waltham, MA, USA). The chromatographic process was performed at room temperature in a gradient manner using the two HPLC methods. Method no. 1, which was used for the analysis of the free BPA in the sheep's urine, pumped the mobile phase of H_2O (constituent A) and MeCN (constituent B) at a flow rate of 1.0 mL/min in the following volume ratios: time 0–2 min (35% B), time 2–12 min (gradient 35–50% B), time 12–20 min (50% B), time 20–20.5 min (gradient 50–35% B), time 20.5–21 min (35% B).[26] HPLC method no. 2, which was used for the analysis of the total BPA in the sheep's urine and of the both free and total BPA in sheep's feces, pumped the mobile phase at a flow rate of 0.9 mL/min and used the two constituents of the mobile phase, i.e., H_2O (constituent A) and MeCN:MeOH = 1:1 (v/v) (constituent B) in the following volume ratios: time 0–2 min (35% B), time 2–22 min (gradient 35–60% B), time 22–35 min (60% B), time 35–40 min (gradient 60–75% B), time 40–42 min (gradient 75–35% B) and time 42–43 min (35% B).[27] For both HPLC methods the excitation and emission wavelengths of the fluorescence spectrophotometry analysis were set at 230 and 315 nm, respectively.[26] The results were evaluated according to an external standard method using a solvent standard calibration curve, which was constructed by plotting the peak area as a function of the analyte concentration. The measured BPA concentration in the baseline sample, taken just before the first administration of BPA to the sheep, was subtracted from all measured samples in the series (study and spiked samples—these were used to calculate recovery of the series). The baseline corrected study sample concentrations (C_{corr}) were then additionally corrected for the mean recovery rate of the series (rec_{mean}, %) as follows: $C = C_{corr}/rec_{mean}*100$. As they were measured in parallels, the mean value was used as a final result.

Quality assurance procedures

Each study sample series consisted of a baseline sample, the study samples (in duplicate) and two recovery samples. These were obtained by fortification of the baseline sample with BPA at a reasonable level. Daily standard calibration curves were constructed from 6 or 7 calibration points. The baseline concentrations for the first, dietary part of the experiment were, in the urine sample, 0.5 μg/L and <10 μg/L, and in feces sample, <1 μg/kg and <1 μg/kg of free and total BPA, respectively. The baseline concentrations for the second, subcutaneous part of the experiment were, in urine sample, 0.15 μg/L and <10 μg/L, and in feces sample, <1 μg/kg and <1 μg/kg of free and total BPA, respectively.

Efficiency of the SPE step was included within the recovery control over the whole procedure, which included extraction from the matrix, SPE and concentration step. Recovery samples were spiked at the beginning of the procedure with the free BPA, also for determination of the total BPA. The recovery was calculated as follows:

$$\text{Rec } (\%) = \frac{(\text{BPA}_{\text{found}} - \text{BPA}_{\text{baseline}})}{\text{BPA}_{\text{added}}} \times 100\%$$

Rec … recovery (%)

BPA_{found} … BPA found in the spiked sample (μg/L or μg/kg)

$BPA_{baseline}$ … BPA found in the baseline sample (μg/L or μg/kg)

BPA_{added} … BPA added in the spiked sample (μg/L or μg/kg)

Validation of BPA analysis

The analytical methodology was validated to demonstrate its fitness for determination of the time profile of BPA excretion in sheep's urine and moist feces. Validation was done separately for free and total BPA in both matrices investigated. Linearity was determined on both a standard and a matrix level by the least squares method, giving the regression and correlation parameters of the calibration lines. Solvent standard concentrations ranged for HPLC method no. 1 from 1.0–50 ng/mL with 6 concentration points per calibration line, while for HPLC method no. 2 they ranged

within 1.5–200 ng/mL with 4–8 concentration points per calibration line. Linearity in matrix, recovery, and precision of BPA determination in sheep's urine and feces were evaluated by fortification of a baseline sample. Linearity on a matrix level was evaluated as an intra-day correlation between the mean measured and added BPA concentrations (n = 2/fortification level), for free and total BPA in urine over a concentration range of 0.5–20 μg/L and 100–15,000 μg/L, respectively, and for both free and total BPA in moist feces over a concentration range of 2–50 μg/kg. Recovery, repeatability and intra-laboratory reproducibility were tested on two BPA concentration levels. Urine samples for determination of the free and total BPA were fortified by 5 and 10 μg BPA/L, and 500 and 2000 μg BPA/L, respectively. Feces samples for determination of both free and total BPA were fortified by 5 and 10 μg BPA/kg. Repeatability was evaluated by 2–5 fortified replicate samples per concentration, tested on the same time occasion, while intra-laboratory reproducibility was evaluated by in total 6–11 fortified samples tested on the 2–4 separate time occasions. The precision of the methods was evaluated using the standard deviation (SD) and coefficient of variation (CV) of the determined values, and assessed in accordance with the Horwitz coefficients (CV_H) according to the European Commission Decision 2002/657/EC.[28] The limit of detection (LOD) value was estimated as the minimum detectable amount of BPA from matrix samples with signal-to-noise ratio of 3:1 and was corrected for the baseline matrix response.

Results

Performance characteristics of BPA analysis

The analytical methodology presented in this work comprises four separate assays for particular BPA category/matrix combinations, and is used for the determination of free BPA in urine/feces and total BPA in urine/feces, which varied in the sample preparation and clean-up, followed by the HPLC analysis. Representative HPLC chromatograms of determining free BPA in sheep's urine and total BPA in sheep's feces are presented in Figs. 2 and 3, demonstrating an appropriate chromatographic resolution and a BPA retention time of around 8.0 and 15.5 min, respectively.

The analytical HPLC methodology demonstrated good linearity, as shown by the obtained correlation coefficients. The "R-squared" values of the solvent standard calibration lines for HPLC method no. 1 over a concentration range of 1–50 ng/mL were ≥ 0.9993, whereas for HPLC method no. 2 over and within a concentration range of 1.5–200 ng/mL they were ≥ 0.9983 (Table 3). The linearity of determining the level of BPA in both matrices is presented in Fig. 4, based on the correlation curves between the found and added values of BPA in sheep's urine and moist feces. The "R-squared" values were 0.9987 and 0.9982 for the free and total BPA in urine, respectively, and 0.9941 and 0.9918 for the free and total BPA in feces, respectively.

The recovery and precision of the method are presented in Table 4 and were determined on two levels of content for particular BPA category/matrix combinations. The recovery values for determination of BPA in urine and feces ranged from 52 to 67% and from 41 to 81%, respectively. The repeatability and within-laboratory reproducibility of the measurements, represented by the CV values, ranged from 1.3 to 27.4% and from 8.8 to 32%, respectively. Regarding urine, the estimated LOD values for determination of free and total BPA were 0.1 and 10 μg/L, respectively. With regard to the feces analysis, the difference in the chromatographic background was smaller between the free and total BPA analysis, resulting in equal LOD value of 1 μg/kg for both free and total BPA, respectively.

BPA, total BPA and BPA–GLUC excretion in urine and feces of sheep after dietary and subcutaneous administration

The free BPA, total BPA and BPA–GLUC concentrations in urine and free BPA, total BPA and BPA–GLUC concentrations in the feces of the sheep after dietary and subcutaneous administration are presented in the Tables 5 and 6, respectively.

After the first day of both experiments, only the trough concentrations were measured in urine each following day, with the exception of the sampling on the fifth day of the dietary and subcutaneous part of experiment, when urine was also taken 8 hours (Table 5) and 7 hours (Table 6) after the last BPA administration, respectively. Another exception was the additional feces sampling which occurred 12 hours after the second BPA administration in the second, subcutaneous part of the experiment (Table 6).

The results in Table 5 demonstrate that there was a very small amount of free BPA excreted in the urine, and that the administered BPA was mostly excreted as BPA–GLUC. On the first day, Cmax (16.6 μg/L) for free BPA was reached after 8 hours of BPA administration and Cmax for BPA–GLUC (15,141 μg/L) was reached after 4 hours. From the end of BPA administration, on the 5th day, the concentrations of free BPA and BPA–GLUC began to decline sharply and got under the analytical LOD for both total BPA and BPA–GLUC on the 8th day, which was three days after the last BPA administration.

Regarding the concentrations of free BPA and total BPA in sheep's feces, Tables 5 and 6 demonstrate that the concentrations of the parent compound (free BPA) were approximately the same as of the total BPA or slightly higher after subcutaneous determination, presumably due to the variability of the analytical method used. Nevertheless, the results demonstrated a general absence of BPA–GLUC in sheep's feces samples irrespective of the form of BPA administration used. Thus, BPA was preferably excreted in feces in its free (aglycone) form.

The results in Table 6 also demonstrate that, as with dietary administration, the BPA in urine was mostly excreted as the BPA–GLUC. On the first day, Cmax for both free BPA

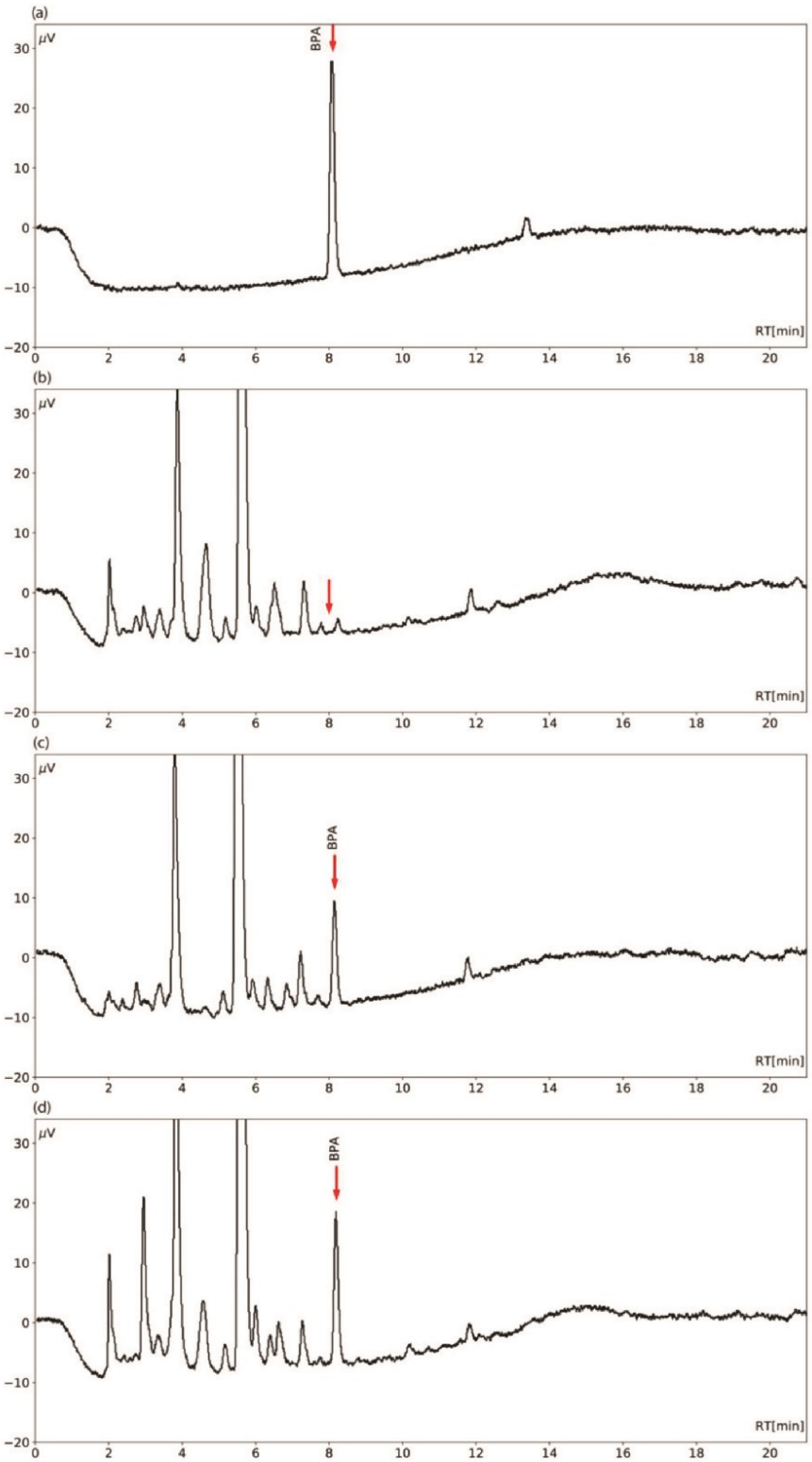


Figure 2. Representative HPLC chromatograms for the determination of free (aglycone) bisphenol A (BPA) in the urine of one sheep: (a) standard solution of 25 ng BPA/mL (b) baseline sample (before BPA administration) (c) baseline sample spiked with 10 μg BPA/L (d) sample containing 15.8 μg BPA/L, obtained 4 hours following p. o. administration to the ewe at a dose of 100 μg BPA/kg b. w.

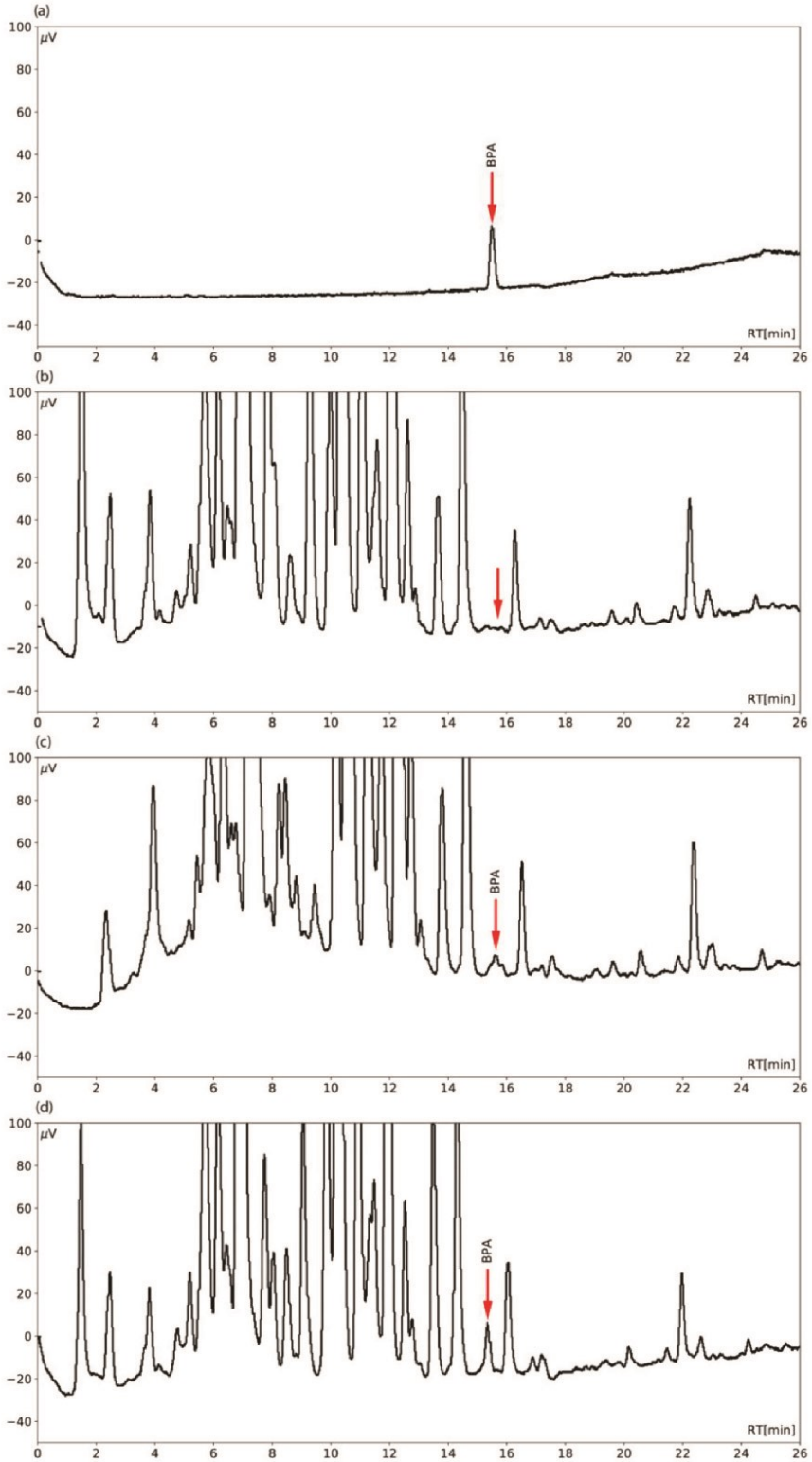


Figure 3. Representative HPLC chromatograms for the determination of the total (free + conjugated) bisphenol A (BPA) in the fresh feces of one sheep: (a) standard solution of 25 ng BPA/mL (b) baseline sample (before BPA administration to the ewe) (c) baseline sample spiked with 10 μg BPA/kg (d) sample containing 34.9 μg BPA/kg, obtained 12.5 hours following p. o. administration to the ewe at a dose of 100 μg BPA/kg b. w.

Table 3. Linearity of bisphenol A (BPA) standard calibration curves obtained by correlation between area of chromatographic peaks and concentration (ng/mL).

HPLC method* (run time)	Concentration range (ng/mL)	Points/curve (no. of curves)	Linear correlation	
			[a]r	r^2
No. 1 (21 min)	1 to 50	6 (9)	0.9996–0.9999	0.9993–0.9999
No. 2 (43 min)	1.5 to 25	4 (1)	0.9973	0.9945
	1.5 to 50	6 (11)	0.9992–1.0000	0.9984–1.0000
	1.5 to 200	8 (1)	0.9991	0.9983
	2.5 to 100	5 (1)	0.9997	0.9994
	2.5 to 200	6 (3)	0.9992–0.9999	0.9983–0.9998
	5 to 200	6 (1)	0.9994	0.9988

[a]correlation coefficient
*HPLC methods are described under Analytical method.

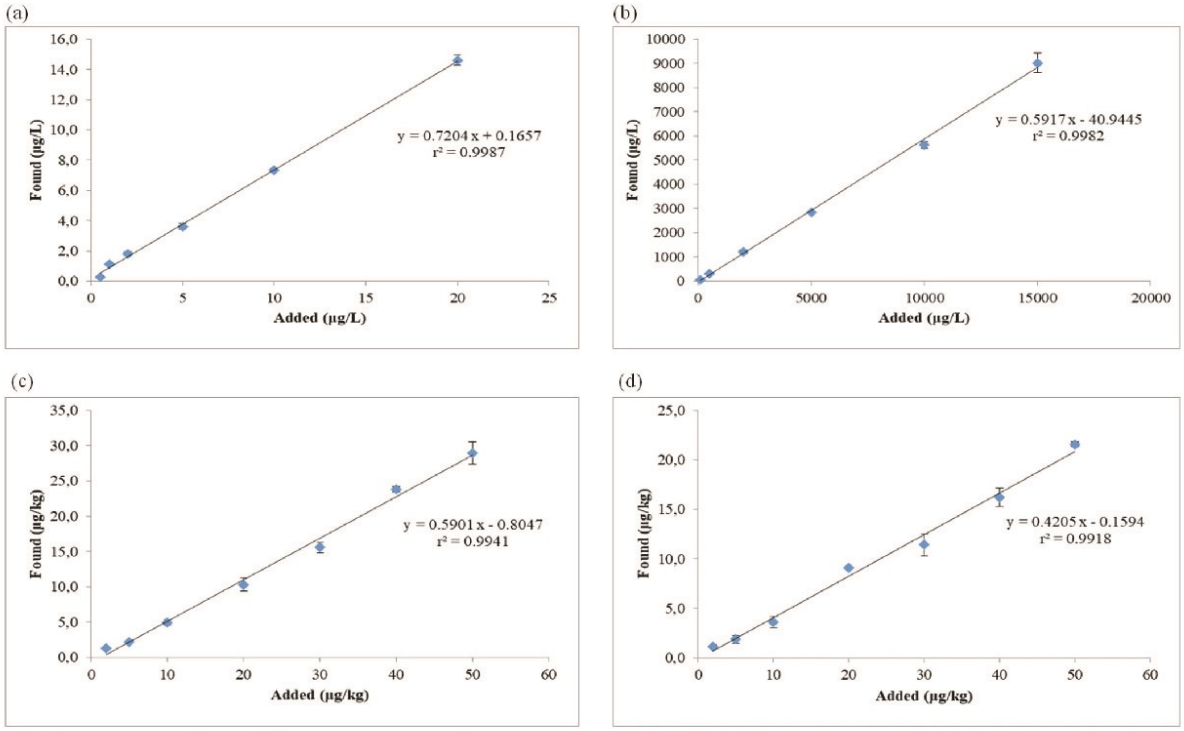


Figure 4. Linearity of analytical HPLC determining the level of bisphenol A (BPA) in sheep's urine and fresh feces, evaluated as an intra-day correlation between the mean measured and added BPA concentrations (n = 2/fortification level); difference bars of both parallels are also presented: (a) free (aglycone) BPA in urine, fortification range from 0.5–20 µg/L (b) total (free + conjugated) BPA in urine, fortification range from 100–15,000 µg/L (c) free (aglycone) BPA in feces, fortification range from 2–50 µg/kg (d) total (free + conjugated) BPA in feces, fortification range from 2–50 µg/kg.

(11.5 µg/L) and BPA–GLUC (13,834 µg/L) was reached after 4 hours of BPA administration. From the last administration of BPA on the 5th day, the concentrations of free BPA and BPA–GLUC were declining sharply and they fell below the analytical LOD on the 7th day, being the 2nd day after the last BPA administration.

Discussion

Development and performance of BPA analysis

An analytical procedure was developed in this work for determination of the time profile of BPA excretion in sheep's urine and feces. Optimizations of the BPA analysis were made mainly regarding the enzymatic deconjugation, extraction from the matrix, concentration of the extract and chromatographic separation.

The aliquot mass of the feces samples was optimized according to the capacity of the SPE Chromabond HR-X, 200 mg cartridges. Therefore, preliminary testing was done taking from 0.25 to 1.5 g of the sample mass. The results clearly demonstrated that sample mass of 1.5 g exceeded the sorbent capacity, as only 26% recovery was obtained. The optimal mass of weighted feces, which enabled as low LOD as possible, was 0.5 g, which gave an average recovery of 51%.

Regarding the deconjugation step, high quality certified reference enzyme with defined activity was used and was for any case added in excess volume of about 2,000 U of the ß-glucuronidase per sample of 0.5 mL of urine compared to

Table 4. Limit of detection (LOD), recovery, repeatability and intra-laboratory reproducibility of bisphenol A (BPA) determination in sheep's urine and moist feces.

Matrix	BPA_{tested}	LOD	Precision category	Fortification level	No. of samples	Mean found ± SD	[a]$Recovery_{mean}$ (%)	CV (%)	[b]CV_H (%)
Urine	Free (aglycone)	0.1 μg/L	Repeatability	10 μg/L	5	5.99 ± 0.49 μg/L	59.9	8.2	32
				5 μg/L	5	3.04 ± 0.32 μg/L	60.7	10.6	36
			Intra-laboratory reproducibility	10 μg/L	11	6.72 ± 0.87 μg/L	67.2	13.0	32
				5 μg/L	10	3.01 ± 0.27 μg/L	60.3	9.0	36
	Total (sum of free + conjugated)	10 μg/L	Repeatability	2,000 μg/L	5	1,215.3 ± 22.3 μg/L	60.8	1.8	14
				500 μg/L	2	313.6 ± 4.0 μg/L	62.7	1.3	18
			Intra-laboratory reproducibility	2,000 μg/L	8	1,037.7 ± 132.5 μg/L	51.9	12.8	14
				500 μg/L	6	289.4 ± 25.3 μg/L	57.9	8.8	18
Feces	Free (aglycone)	1 μg/kg	Repeatability	10 μg/kg	5	5.09 ± 0.89 μg/kg	50.9	15.9	32
				5 μg/kg	5	2.03 ± 0.56 μg/kg	40.7	27.4	36
			Intra-laboratory reproducibility	10 μg/kg	8	6.30 ± 1.82 μg/kg	63.0	28.9	32
				5 μg/kg	7	2.08 ± 0.48 μg/kg	41.6	23.1	36
	Total (sum of free + conjugated)	1 μg/kg	Repeatability	10 μg/kg	5	8.06 ± 1.09 μg/kg	80.6	13.5	32
				5 μg/kg	4	2.96 ± 0.56 μg/kg	59.2	19.1	36
			Intra-laboratory reproducibility	10 μg/kg	7	6.76 ± 2.16 μg/kg	67.6	32.0	32
				5 μg/kg	8	2.82 ± 0.47 μg/kg	56.3	16.7	36

[a]recovery was determined on the basis with free BPA spiking.
[b]Horwitz coefficient of variation.[28]

Table 5. Detected free, total and BPA–GLUC concentrations in urine and feces samples after dietary BPA administration of 100 μg/kg b. w./day to an ewe.

			BPA concentration				
			Urine (μg/L)			Feces (μg/kg)	
Day	Daily BPA adm.*	Hours after BPA adm.	Free BPA	Total BPA	BPA–GLUC**	Free BPA	Total BPA
1	YES	1 h	4.51	8,385	8,380	/	/
		3 h	/	/	/	<1.00	<1.00
		4 h	15.81	15,157	15,141	<1.00	<1.00
		6 h	12.86	7,368	7,356	/	/
		8 h	16.55	6,434	6,417	/	/
		10 h	5.15	4,811	4,805	/	/
		12 h 30 min	3.25	3,967	3,964	24.41	34.93
2	YES	22 h 20 min	1.60	2,225	2,224	17.41	32.56
3	YES	22 h 15 min	2.11	1,643	1,641	/	/
4	YES	22 h 46 min	1.43	2,196	2,195	45.25	53.40
5	YES	23 h	1.09	1,058	1,057	18.89	19.19
		8 h	1.94	2,577	2,576	/	/
6	NO	23 h 45 min	1.38	1,022	1,021	25.23	49.01
7	NO	2 days	0.79	17.88	17.09	3.96	4.93
8	NO	3 days	0.41	<10.0	<10.0	<1.00	<1.00

*adm. is referring to administration.
**BPA–GLUC is calculated as the difference between total BPA and free BPA, as the BPA–GLUC was determined indirectly by enzymatic conversion of the BPA–GLUC to free BPA.
/not tested.

the reported values by literature.[29,30] As recovery samples for determination of the total BPA were also spiked at the beginning of the procedure with the free BPA due to aggravated availability of the certified reference BPA–GLUC, we did our own testing to control and optimize the time duration of the deconjugation process. We took a real study urine sample taken at 4 hours post p. o. administration of BPA at a dose of 0.1 mg/kg b. w. to the ewe and performed the enzymatic deconjugation within different incubation time periods, which justified the time of 16–17 hours, taken for the analysis of urine, being also in line with references in the literature.[24,29,30,31] Regarding feces, the incubation time was shortened to 4 hours due to significantly lower expected concentrations in the study samples. At the end of incubation, the feces samples were totally decomposed as a result of an extremely effective enzymatic activity of the β-glucuronidase used. Additionally, the BPA standard for recovery testing of the total BPA was diluted preferably in H_2O, with an MeCN share of 0–0.25% (v/v), which prevented the denaturation of the enzyme used. This is in accordance with Markham et al.,[30] who demonstrated that the enzyme viability is affected when the organic level exceeds 0.5% of the sample volume, particularly at lower concentrations (<2 ng/mL).

While urine samples were applied directly onto the SPE cartridge, after dilution with water or sodium acetate buffer, BPA from feces had to be extracted using an organic solvent. MeCN was used because of its excellent solubility for the BPA and its strong ability to denature enzymes and precipitate proteins to produce an acceptable matrix background. Re-extraction was also included to increase effectiveness, and no hydrolysis was observed. The following two SPE steps were carried out using modern sorbent materials, prepared with advanced technology, and this is the spherical, hydrophobic polystyrene–divinylbenzene (PS/DVB) copolymer Chromabond HR-X and molecularly

Table 6. Detected free, total and BPA–GLUC concentrations in urine and feces samples after subcutaneous BPA administration of 100 μg/kg b. w./day to an ewe.

			BPA concentration				
			Urine (μg/L)			Feces (μg/kg)	
Day	Daily BPA adm.*	Hours after BPA adm.	Free BPA	Total BPA	BPA–GLUC**	Free BPA	Total BPA
1	YES	1 h	3.42	5,000	4,996	/	/
		2 h	4.24	8,683	8,679	/	/
		4 h	11.53	13,846	13,834	/	/
		4 h 30 min	/	/	/	<1.00	<1.00
		5 h	/	/	/	<1.00	<1.00
		6 h	0.43	3,262	3,261		
		6 h 15 min	/	/	/	4.14	4.41
		8 h	0.63	2,578	2,577	<1.00	<1.00
		10 h	0.88	1,602	1,601		
2	YES	23 h 55 min	0.60	<10.0	<10.0		
		12 h	/	/	/	3.36	2.72
						3.94	3.39
3	YES	23 h 55 min	0.10	15.63	15.52	/	/
4	YES	23 h 25 min	0.41	/	/	26.65	26.51
5	YES	22 h	1.21	456.0	454.8	22.45	20.98
		7 h	2.91	1,723	1,720	/	/
6	NO	23 h 25 min	0.22	18.13	17.91	8.27	5.32
7	NO	2 days	<0.1	<10.0	<10.0	<1.00	<1.00
8	NO	3 days	<0.1	<10.0	<10.0	<1.00	<1.00

*adm. is referring to administration.
**BPA–GLUC is calculated as the difference between total BPA and free BPA, as the BPA–GLUC was determined indirectly by enzymatic conversion of the BPA–GLUC to free BPA.
/not tested.

imprinted polymer (MIP) AFFINIMIP® SPE Bisphenols. The two SPE steps basically followed the analytical method of Deceuninck et al.,[25] developed for the analysis of the free BPA in a large set of food items. However, the final derivatisation step, intended for gas chromatography tandem mass spectrometry (GC-MS/MS) determination, as used in their method, was omitted due to the HPLC-fluorescence analysis used by the method presented in this work. The final sample extracts were dissolved in 0.5–1.0 mL of the starting HPLC mobile phase, containing 35% (v/v) MeCN in H_2O, which had to be additionally diluted (5–25 fold) in the case of analysis of the total BPA in urine to achieve the chromatographic results within the standard calibration curve used in this study.

We did not observe matrix effect due to physicochemical specificity of the fluorescence detection (for the difference of the mass spectrometric detection where such an effect is frequently reported). The greatest complexity of the chromatographic background was observed by urine analysis, preferably of the total BPA, resulting in a 100-fold higher LOD concentration level than for the free BPA. Nevertheless, the composition of the organic component in HPLC method no. 2, comprising MeOH and MeCN in a volume ratio of 1:1 according to Petersen et al.,[32] significantly improved the chromatographic selectivity of BPA from the comprehensive matrix background, as observed for the total BPA in urine and both free and total BPA in the feces.

The importance of appropriate quality control of sample testing was considered, as baseline controls, fortified controls, and study sample replicates were included with each analysis set to avoid and minimize possible artifacts or contamination and ensure appropriate performance characteristics of the BPA analysis. In addition, storage devices with declared absence of BPA were used, high quality glassware was used where possible, and the solvents used in the study were mainly of HPLC grade and screened via reagent blanks.

The analytical procedure for the determination of the total BPA was more demanding than for the determination of only free BPA due to the deconjugation step within the enzymatic decomposition of the sample, which consequently gave a more comprehensive matrix background, and influenced the validation parameters. The moderate recovery levels in sheep's urine and feces, ranging from 52 to 67%, and from 41 to 81% (Table 4), were a consequence of a comprehensive clean-up, including two SPE purification steps needed for isolation of the analyte from the complex biological matrices. Moreover, these moderate recovery levels were absolute levels, as an internal standard was not used, and this is a difference with the LC-MS/MS methods reported in the literature,[12,17,21,23] with these methods also used in human biomonitoring studies.[29,30,33] The repeatability and within-laboratory reproducibility yielded with the CV values, ranging from 1.3 to 27.4% and from 8.8 to 32%, respectively, was generally higher for feces than for urine analysis due extraction of BPA from feces, but did not exceed the CV_H values from the Horwitz equation.[28] Moreover, in 75% of cases the CV values were below two-thirds of the corresponding CV_H values, and thus fully acceptable (Table 4). The analytical LOD value of 0.1 μg/L for free BPA in urine was the same as reported by Zhang et al.,[11,12] while the reported LOD values for total BPA by LC-MS/MS[21,23] were two concentration orders of magnitude lower than the reported value of 10 μg/L obtained by our method, which was a consequence of the better sensitivity and selectivity of mass spectrometric detection in comparison with fluorescence detection at emission and excitation wavelengths below/around 300 nm for the analysis of very complex biofluids. Regarding feces, our LOD value

of 1 μg/kg obtained for both free and total BPA was in the same concentration order of magnitude as the values for BPA of 5 μg/kg and BPAF of 3 μg/kg, being reported by Twaddle et al.[23] and Yang et al.,[24] respectively.

Excretion of BPA, BPA–GLUC and total BPA with urine and feces from an experimentally dosed sheep

Based on the appropriate performance characteristics of the analytical method, we tested BPA excretion in one experimentally dosed sheep. Our study has been one of the few to measure the BPA in the samples of urine in a sheep model,[17,21,34] and to the best of our knowledge no experimental studies were conducted on a sheep model to analyze the excretion of BPA in feces. In our work both dietary and subcutaneous administrations were performed. As biological samples and experimental settings are highly valuable nowadays due to the 3 R (replacement, reduction, refinement) principle, as laid down by the Directive 2010/63/EU of the European Parliament and Council on the protection of animals used for scientific purposes,[35] the detected concentrations of BPA in the urine and feces after subcutaneous administration are reported in this paper as well, even though farm animals would rarely be exposed to subcutaneous administration of BPA. Unfortunately, as neither a metabolic cage nor catheter was used in the experiment, the samples of urine and feces were not total, and thus the percentage of the administered dose was not calculated to omit poor estimation.

The results in our study are comparable with those of studies performed on sheep,[17,34] monkeys[19] and pigs,[20,36] as the BPA in our study was mostly excreted in urine as BPA–GLUC and only a small fraction was excreted in urine as free BPA. Nevertheless, it is important to state that in our work the concentrations of BPA–GLUC in urine were probably slightly overestimated, as the enzyme ß-glucuronidase from *Helix pomatia* Type HP-2 was used for total BPA determination, containing both glucuronidase (≥100,000 U/mL) and sulfatase (≤7,500 U/mL) activity. The overestimation of BPA–GLUC obtained with enzymatic deconjugation was reported by Lacroix et al.,[17] who measured BPA–GLUC directly and compared its concentration with the results of enzymatic deconjugation of BPA–GLUC. As stated above, BPA has been mostly excreted through the kidneys as BPA–GLUC not only in sheep, but in monkeys and pigs as well. In rats, however, the fraction of total BPA excreted in urine was much lower,[18] due to the suggested enterohepatic recirculation of BPA, which was consequently excreted with feces. Regarding other farm animal species, however, one would speculate that the excretion route as reported in sheep is similar for all ruminants (cows, goats, etc.) due to the similarities in their gastrointestinal tracts. To the best of our knowledge, there is no research done on horses and other equids or poultry, and only two studies were conducted on pigs,[20,36] where the researchers reported that after oral dosing BPA was predominately excreted as BPA–GLUC, and approximately half of the dose was excreted 3 hours after administration.[36]

Experimental studies in which BPA was determined in the feces of animal models are even rarer. To the best of our knowledge, such studies were only performed on monkeys[19] and rats.[18] In the feces samples of the monkeys, a much smaller fraction of the dose was excreted via the feces than in rats. Nevertheless, in both species, monkeys and rats, only free BPA was detected in the samples. In our study, the detected free BPA concentrations in feces were similarly low as in monkeys. Interestingly, in our work the total BPA concentrations were approximately of the same levels as found for free BPA, meaning there is a general absence of BPA–GLUC in the sheep's feces samples irrespective of the form of BPA administration, and that BPA is preferably excreted in feces in its free form. However, the concentrations of free BPA in feces at some sampling points were slightly higher then of total BPA after subcutaneous determination, presumably due to variability of the analytical method used, yet hydrolysis in these samples can not be ruled out.

Beside experimental studies, a couple of field studies were conducted, in which the samples of fresh urine, fresh feces, manure or liquid manure were taken directly from the farms.[11–16] In Table 2, the determined concentrations in urine and feces from the field studies are reported. It can be seen that the reported concentrations of free BPA in our study (<1–45.25 μg/kg for feces and <0.1–16.55 μg/L for urine) are in the same concentration range as the reported concentrations in urine and feces[11,12] but generally lower than the concentrations determined in manure samples.[13–16]

In Zhang et al. BPA was found in urine samples in the range from 218 to 446 ng/L[11] and from 1 to 2,120 ng/L[12] and in feces samples in the range from not detected (nd) to 13 μg/kg[11] and from nd to 4 μg/kg.[12] Zhang et al. believed that the BPA found in the samples of urine and feces most likely originated from materials used to coat the inner surfaces of animal food containers.[11] In Kinney et al. BPA was not detected in swine manure,[16] while in Aznar et al. BPA was detected in poultry manure at levels up to 207 μg/kg[13] and in Xu et al. it was detected in hen, duck and swine manure at levels up to 167 μg/kg, 179 μg/kg and 362 μg/kg, respectively, while the concentrations of BPA were lower only in cow manure and were at levels up to 33 μg/kg.[15] Regarding liquid manure, in Fromme et al. the BPA levels ranged between 61 and 1,112 μg/kg of dry weight (d. w.).[14] The authors believed that BPA presence in the liquid manure was most likely the consequence of migration from the inner surface coating of the manure tanks, yet they speculated that contribution entering via animal feed could not be ruled out. It is important, however, to consider that in Fromme et al.[14] the researchers measured BPA based on dry weight, and thus the BPA in dried samples was very concentrated. Hence, it is not relevant to compare their results with other research.

Interestingly, in all the field studies only the free BPA was measured in the fresh urine, fresh feces and manure samples. That seems relevant for fresh feces, as presumably there is only free BPA excreted in it. However, it is assumed

that, like natural estrogens, BPA is deconjugated prior degeneration,[37] and it was found that in humans, monkeys, sheep and pigs BPA is predominantly excreted in urine as its main metabolite, with only a small fraction excreted as free BPA.[17,19,20,38] It is important to be aware of this when using detected concentrations in risk assessments, as concentrations of free BPA could be higher or lower depending on the time of sampling. That is especially true for urine, meanwhile in manure it also depends on the type of manure (liquid or not) and the content of urine in it. In addition, it depends on many other physiological, microbiological and chemical factors, which influence the sample. Thus, it would be of great help, if researchers specified the collection protocol and approximate composition of the manure in the future studies.

Conclusion

The analytical strategy presented in this work enabled the analysis of both free and total BPA in urine and feces samples from a biological experiment by using HPLC-fluorescence technology, which evaluated the BPA concentration profiles by both dietary and subcutaneous administration to one ewe. The results obtained in this work show that the method could also be applied to other ecotoxicological studies of BPA, BPA–GLUC and total BPA in urine and feces. There is currently not much research devoted to the testing of BPA in animal excreta, nor animal feed, although the ingestion of BPA contaminated feed might contribute to the burden of BPA in our environment.

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2.3 BAZIČNA RAZISKAVA DIETARNEGA VNOSA BISFENOLA A (BPA) OVNOM PASME ISTRSKA PRAMENKA IN UGOTAVLJANJE REPRODUKTIVNE TOKSIČNOSTI

Basic exploratory study of bisphenol A (BPA) dietary administration to Istrian Pramenka rams and male toxicity investigation

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Izvleček

BPA je okoljsko onesnaževalo, ki povzroča endokrine motnje. Po poročanju raziskovalcev ima BPA vpliv na reproduktivno toksičnost različnih živali v postopkih. V tej raziskavi smo ugotavljali, ali dolgotrajna dvomesečna izpostavljenost 25 µg/kg t. m. (majhen odmerek) BPA vpliva na spermatogenezo ali kakovost sperme pri mladih ovnih pasme istrska pramenka, ki so bili BPA izpostavljeni s hrano. Ocenili smo telesno maso in maso mod, histopatologijo mod in nadmodkov ter analizirali semenčice. Čeprav je bilo med obema skupinama nekaj razlik, te niso bile velike ali statistično značilne. Edina statistično značilna razlika je bila nižja višina zarodnega epitela zvitih semenskih cevk pri tretiranih ovnih v primerjavi s kontrolnimi ovni. Poleg ugotavljanja toksičnosti so bile po prvem dajanju določene koncentracije BPA v krvni plazmi tretiranih ovnov in izračunani toksikokinetični parametri celokupnega BPA. V tej raziskavi nismo odkrili večjih sprememb, ki bi nakazovale na motnje v reprodukciji pri ovnih.

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Basic Exploratory Study of Bisphenol A (BPA) Dietary Administration to Istrian Pramenka Rams and Male Toxicity Investigation

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Abstract: Bisphenol A (BPA), an endocrine-disrupting chemical and environmental pollutant, has been reported by many researchers to induce male reproductive toxicity in different experimental models. In this study, we investigated whether long-term exposure for two months to 25 µg/kg body weight (low dose) of BPA affects spermatogenesis or sperm quality in young Istrian Pramenka rams exposed via diet. We evaluated body and testicular weights, histopathology of testes and epididymides, and sperm analyses, and compared these parameters between the group of treated rams and the control group of rams. Although there were some differences between the two groups, these differences were not large or statistically significant. The only statistically significant difference was the lower epithelial height of seminiferous tubules in treated rams, compared to control rams. In addition to assessing toxicity, BPA concentrations in the blood plasma of treated rams were determined after the first administration, and the toxicokinetic parameters of total BPA were calculated. In this study, no major signs of altered reproduction in rams were detected.

Keywords: bisphenol A; rams; toxicokinetics; male toxicity; sperm

1. Introduction

Many synthetic chemicals that are ubiquitous in our environment can have negative effects on the health of wildlife, domestic animals, and humans. Some of them can bind to and disrupt endogenous hormone receptors and are therefore classified as endocrine-disrupting compounds (EDCs) [1].

One of these chemicals is bisphenol A (BPA), a substance produced in large quantities and used in a variety of common consumer products, including food and beverage packaging, flame retardants, adhesives, building materials, electronic components, and paper coatings [2]. The weak estrogenic, anti-androgenic, and anti-thyroid effects of BPA, combined with the increasing incidence of reproductive disorders observed over the past decade, have led to health concerns about human exposure to BPA [3]. Considering health concerns for humans, the European Food Safety Agency (EFSA) established the tolerable

daily intake for BPA to 50 μg/kg bw/day, which was subsequently lowered to 4 μg/kg bw/day in 2015 [4]. Furthermore, in the current re-evaluation and draft opinion, the EFSA is considering reducing the TDI even further (0.04 ng/kg bw/day) [5].

To investigate the adverse effects of BPA on the male reproductive tract, numerous in vivo reproductive toxicity studies have been conducted in laboratory rodents [6]. In vivo studies have been conducted primarily during pregnancy or lactation, as these periods appear to be particularly sensitive windows of exposure [7]. Based on occupational exposure of workers from BPA-producing and processing factories, and on epidemiological studies showing the deterioration of semen characteristics in these workers [8], concerns have been raised about whether BPA could affect male reproductive health when exposure occurs during later phases of life, such as puberty or adulthood. Additional in vivo studies have been conducted in adult rodents, and more than thirty of these studies have resulted in mixed outcomes. Effects were or were not detected within a wide range of doses (0.0002 to 960 mg/kg of body weight (bw)) [9,10], exposure periods (6 days–48 weeks) [11,12], administration routes (drinking water, gavage, subcutaneous, intraperitoneal injection) [12–15], and species of rats (Wistar, Sprague–Dawley, albino) [16–18] or mice (Kunming, Pzh:SFIS outbred laboratory, CD-1 mice, Swiss-albino mice, ICR mice, C57BL/6 mice) [12,19–23]. Briefly, in BPA-treated animals, absolute and/or relative reproductive organ weights were decreased [9,10,18,24–27], or the testes weights were even increased [11]. In some BPA-exposed rodents, a smaller seminiferous tubule diameter [10,26,28], a lower epithelial height of seminiferous tubules [11,16,29], or higher numbers of apoptotic cells [19,21,30,31] were detected. Histopathologically, a decrease in the number of Leydig cells [10,24], the degeneration of Leydig cells and germ cells [27], a reduced number of germ cells [19,21], germ cell exfoliation [12,28], necrosis of germinal epithelium [32], and cytoplasmic vacuolation [29] were reported in the testes of examined animals. Regarding sperm quality and quantity, BPA was reported to decrease epididymal sperm motility, sperm count, sperm production, sperm reserves, and sperm transit time, and to increase sperm fragility and sperm DNA damage [9,14,17,29,33,34]. Furthermore, BPA disturbed the pro-oxidant–antioxidant balance of testicular and epididymal tissue of adult rats [9,17,18,35].

Only two in vivo studies were performed on mammals other than rodents; in one experiment, these animals were common marmosets [36], and in the other, goats [37]. In the latter study, adult male goats were administered BPA (25 mg/kg) as a positive control for the main substance studied—a plant extract, *Ipomoea carnea*. The only lesion noted in BPA-exposed bucks was vacuolar degeneration in the rete testis and the decreased integrity of the plasma membrane of the spermatozoa [37].

Uncertainties remain regarding the toxic potential of BPA on male reproduction in mammals and the relevance of the experimental data to humans. In addition, the experimental data are mainly from specific rodent species and strains, for which varying sensitivity to estrogenic substances has been reported [38]. Furthermore, the most comparable route of exposure for humans would be dietary, since human exposure to BPA is thought to occur mainly via food [39]. This fact is supported by the results of the study by Guignard et al. [40], in which the absolute bioavailability of BPA was about three times higher after dietary administration than after gavage. Therefore, gavage was not considered a better route of exposure than subcutaneous application of BPA [41].

The aim of this multidisciplinary study was to investigate the effects of long-term dietary exposure to a relatively low dose of BPA on the testes, epididymides, and spermatozoa of pubertal rams. During our two-month experimental study, rams in the treated group were fed a diet containing 25 μg BPA/kg bw/day. The dose was chosen on the assumptions of Guignard et al., who suggested that people could ingest several tens of μg BPA per kg per day [40]. Their assumptions of the daily intake were based on pharmacokinetic studies performed on various animals [42] and the commonly described human plasma BPA concentrations in the range of ng/mL [43]. The second aim of this study was to confirm the internal exposure of rams treated with BPA, as internal exposure is rarely reported. Using our analytical method, developed in a previous study on an Istrian

Pramenka sheep [44], we aimed to detect free and total BPA in blood and testicular tissue by enzymatic deconjugation (for total BPA), organic solvent extraction, molecularly imprinted polymer solid-phase extraction (MISPE) clean-up, and high-performance liquid chromatography with fluorescence detection (HPLC-FLU). Blood samples were collected multiple times to evaluate the toxicokinetic profile after the first BPA administration.

2. Materials and Methods

2.1. Chemicals

Bisphenol A ≥ 99% purity (Merck, Sigma-Aldrich, Darmstadt, Germany) was dissolved in absolute ethanol. Solutions were stored at ambient temperature in sealed amber glass bottles for the entire period of use. All materials used for the solution preparation, sample processing, and assays were either glass or BPA-free plastics.

2.2. Animals and Their Environment

The study was conducted on adult Istrian Pramenka rams in a sheepfold at the Infrastructure Centre for Sustainable Recultivation Vremščica of the Veterinary Faculty of the University of Ljubljana, Slovenia. The center is located in a rural area in western Slovenia with a temperate continental climate. The rams were born at the center in the wintertime (birth dates are presented in Supplementary Table S1). They were nine-months old at the beginning of the experiment and weighed 34.5–54 kg. The rams were randomly assigned to two groups, a control group (n = 7) and a treated group (n = 7). Random allocation was performed by physical randomization. The rams were marked with conventional sheep ear tags. A caretaker monitored the rams three times per day. Prior to the experiment, a basic veterinary examination (temperature, respiratory rate, pulse rate, rumination frequency) and a basic blood analysis were performed to ensure that rams were clinically healthy (see Supplementary Tables S2–S4).

Rams were kept indoors, under natural light, temperature conditions (6 to 15 °C), and relative air humidity (45–55%) in pens with wooden and metal grids. One collective pen (2.6 × 4 m) contained the bucks of the control group, and the other pen (2.6 × 4 m) contained the bucks of the treated group. No direct contact was allowed between the animals of the different groups, and separate feed and water supplies were provided for each group. Animals were housed individually during administration and sampling.

Water and hay were available ad libitum, and each morning the rams received vegetable pellets (Schafkorn Lac, Unser Lagerhaus Warenhandels Ges.m.b.H). Feeding management was the same for both groups of rams.

We tested for BPA contamination in water, hay, and pellets, and BPA was not detected in any of the samples. The pens and individual stalls, common water supply (50 L enamel pot), and feeding containers (stainless steel bowls) were all made of either wood, enamel, or stainless-steel to minimize background levels of BPA.

Both groups of rams were euthanized at 11 months of age. The experiment lasted 64 days, which is sufficient for the entire duration of spermatogenesis and for the transition of sperm in the *ductus deferens* in rams. Before euthanasia, rams were premedicated with xylazine (2 mL Xylased 5%, Chanelle Pharmaceuticals Ltd., Loughrea, Ireland, i/v) and euthanized after seven to ten minutes with pentobarbital (Exagon, Richter Pharma, Wels, Austria; 2 mL/10 kg bw).

All animal procedures were performed in accordance with ethical standards and approved by the Administration of the Republic of Slovenia for Food Safety, Veterinary Sector and Plant Protection; approval numbers U34401-3/2015/8 and U34401-3/2015/17. All procedures involving animals and the experiment were in accordance with the Slovenian Animal Protection Act [45], Council Directive [46], and ethical principles.

2.3. BPA Exposure Protocol

The treated group received a daily dose of 25 µg BPA/kg in the diet. Approximately 1 mL of BPA solution in absolute ethanol was applied to the pellet ration to obtain the

single dose of 25 µg/kg body weight, and was administered with the morning feeding of pellets (550 g) that lasted approximately 15 min. A caretaker ensured that rams ate the entire meal each day. The rams were weighed once a week and the administered volume was adjusted to the last recorded body weight. The control group received 1 mL of ethanol without BPA, applied and administered similarly to the BPA-treated group.

Fresh BPA solution was prepared five times during the experiment, and the stability of BPA in ethanol at concentration of 2 mg/mL was proved over 18 days in our previously performed experiment on BPA toxicity in aquaculture.

2.4. Sampling Protocols

2.4.1. Blood Plasma

On the first day of the experiment, blood sampling was performed after BPA administration. Blood samples were collected from the rams at time 0 (before the first administration), and at 0.083, 0.16, 0.33, 0.5, 1, 2, 4, 6, and 8 h. The sampling time began when the rams had consumed the entire portion of the pellets.

Blood samples from the jugular vein were collected in heparinized glass vacuum tubes, cooled to 4 °C, and transported to the laboratory where the blood plasma was separated by centrifugation at 2640× *g* for 15 min. Plasma was transferred to polypropylene tubes (PP) and stored. Plasma samples were frozen at −20 °C until analysis. Blank blood plasma samples were collected from rams before the start of the experiment to provide a baseline for analysis. To avoid contamination with BPA during sampling, glass vacuum tubes were used for blood collection.

2.4.2. Gross Morphology and Processing of Organs

After euthanasia, rams were weighed; then, the left and right testes were removed, weighed, and sampled, and the epididymides were removed.

Cross sections were made in both testes to the approximate midpoint for rapid fixation. After fixation in Bouin's fixative for six hours, a 1 cm-thick block of tissue was excised and fixed for an additional 24 h, rinsed in 70% ethanol, further fixed in neutral phosphate-buffered 4% formaldehyde (10% formalin), further processed, embedded in paraffin wax, sectioned, and stained with a hematoxylin and eosin (H&E) stain and a periodic acid–Schiff (counterstained with hematoxylin) (PAS-H) stain. The complete right epididymis was fixed in 10% buffered formalin, processed, embedded in paraffin, sectioned, and stained with H&E.

The left epididymis was excised, ligated at the *ductus deferens*, individually packed in polyethylene bags, placed on ice, and immediately transported in an ice chest to the laboratory of the Clinic for Reproduction and Large Animals of the Veterinary Faculty of Ljubljana for further processing. After the testes and epididymides were sampled, a complete necropsy was performed on each ram.

2.5. Blood Plasma BPA Determination

Samples of blood plasma were analyzed for the presence of free (aglycone) and total (sum of free and conjugated) BPA. Total BPA was determined in free form by enzymatic deconjugation of the glucuronide bond. Blood plasma samples were extracted and prepared for high-performance liquid chromatography (HPLC) analysis, as described by Šturm et al. [44]. The HPLC analysis, quality assurance procedures, validation of BPA analysis, and performance characteristics of BPA analysis are described in the Supplementary Material S3.

2.6. Toxicokinetics

Toxicokinetic analyses were performed with EquivTest/PK software (Statistical Solution Ltd., Cork, Ireland). The plasma concentration time course from the first BPA administration was analyzed using a non-compartmental approach to obtain the toxicokinetic parameters. The calculated parameters were C_{max}, T_{max}, k_{el}, $t_{1/2}$, AUC, AUMC, MRT, CL, and Vd. The C_{max} was the maximum observed plasma concentration. The T_{max} was

the time of the maximum observed plasma concentration. The k_{el} was the terminal slope of the concentration profile in the semi-log plot calculated by linear regression. The $t_{1/2}$ was the elimination half-life, calculated as the ratio between ln(2) and k_{el}. The area under the curve to the last concentration higher than LOQ (AUC_t) was calculated using the linear trapezoidal method until eight hours, and the area under the curve to the infinity (AUC_i) was sum of the AUC_t and the extrapolated part to infinity by the addition of the term C_{last}/k_{el}, where C_{last} is the last quantified concentration (8 h in this study). The area under the moment curve (AUMC) was the area under the curve of the product of concentration and time versus time. The mean residence time (MRT) was calculated as the ratio between the AUMC and AUC. The clearance (CL) was calculated as the ratio between the dose and AUC_i. Volume of distribution (Vd) was calculated as the ratio between clearance and k_{el}.

2.7. Histopathology of Testes and Epididymides

A histopathological examination of 4 µm-thick tissue sections of formalin-fixed paraffin-embedded (FFPE) samples of testes and epididymides stained with H&E and PAS-H was performed by light microscopy. The examined slides were blinded with regards to the treatment groups. Testes were evaluated in a "stage-aware" manner [47–49]. We based the evaluation of the testes and epididymides on the published recommendations of Lanning et al. [50] and Creasy et al. [51]. The endpoints examined in the testes of rams in our study were perivasculitis, Sertoli-only tubules, segmental hypoplasia, vacuolation of Sertoli cells, multinucleated cells, mononuclear infiltrates, sperm retention, sperm head phagocytosis in the basal Sertoli cell cytoplasm, and sperm granuloma, as well as Leydig cells atrophy, Leydig cells hypertrophy/hyperplasia, and Leydig cells vacuolation. In the rete testis, we examined the following endpoints: mononuclear infiltrates, multinucleated cells, mineralization, and fibrosis. The endpoint examined in the epididymis were mononuclear infiltrates, pyknotic sperm, sloughed epithelial cells and sperm granuloma.

Histopathologic changes were described, wherever possible, according to distribution, severity, and morphological character. Severity scores were assigned on a scale of one to five. Grade 1 (minimal changes) corresponds to a histopathologic change ranging from inconspicuous to barely noticeable, but so minor, small, or infrequent as to warrant no more than the least assignable grade. For multifocal or diffusely distributed lesions, this grade was used for processes where less than approximately 10% of the examined tissue was involved. Grade 2 (slight changes) corresponds to a histopathologic change that is a noticeable but is not a prominent feature of the tissue. For multifocal or diffusely distributed lesions, this grade would be used for processes where between approximately 10 and 25% of the examined tissue would be involved. Grade 3 (moderate changes) corresponds to a histopathologic change that is a prominent but not dominant feature of the tissue. For multifocal or diffusely distributed lesions, this grade would be used for processes where between approximately 25 and 50% of the examined tissue would be involved. Grade 4 (marked changes) corresponds to a histopathologic change that is a dominant but not overwhelming feature of the tissue. For multifocal or diffusely distributed lesions, this grade would be used for processes where between approximately 50 and 95% of the examined tissue would be involved. Grade 5 (severe changes) corresponds to a histopathologic change that is an overwhelming feature of the tissue. For multifocal or diffusely distributed lesions, this grade would be used for processes where more than approximately 95% of the examined tissue would be involved.

A study pathologist examined all slides, which were subsequently peer-reviewed by an internationally accredited toxicological pathology diplomate. Furthermore, a pathology working group (PWG) composed of the reviewing pathologist, a study pathologist, and three additional pathologists reviewed the slides, and the final diagnoses for the reviewed lesions represent a consensus of the PWG.

Testis Histomorphometry

Epithelial height, tubular diameter, and tubular area were measured in seminiferous tubules in the left testis of rams. Digital images of H&E-stained paraffin sections were taken with a Nikon Microphot-FXA (Nikon Digital SightDS-2M) microscope using a 40× objective lens in almost all cases, except for three tubules from the control group and seven tubules from the treated group, which were too large to be captured using a 40× objective lens. Therefore, a 20× objective lens was used to capture these latter tubules. Images were stored as uncompressed TIFF files, at 1200 × 900 pixels and 0.4 µm per pixel, as red–green–blue (RGB) images with eight bits per channel. A total of 245 round-shaped, straight-cut, seminiferous tubules at the stages VII and VIII (shortly after spermiation) were evaluated. The number of tubules evaluated from the control and treated groups was 116 and 129, respectively. The mean (range) number of seminiferous tubules measured from the control group was 16.6 (15–23) tubules per ram, and from the treated group, 18.4 (14–24) per ram. Morphometric measurements of the area, diameter, and epithelial height of seminiferous tubules were analyzed by semi-automatic image analysis, as described in Spörndly-Nees et al. [7]. The measurements were performed blinded to the outcome.

2.8. Semen Analyses

Semen samples were collected using the flotation method from the right *ductus deferens* and the epididymis, which was separated into three parts: head, body, and tail. Semen samples were analyzed for sperm concentration, morphology, motility, and plasma membrane integrity.

In brief, sperm concentration was assessed with an improved Neubauer hemocytometer. Sperm motility was assessed with a computer-assisted semen analyzer (Hamilton Thorne Biosciences, Version 12.3, Beverly, MA, USA) (CASA). Additionally, plasma membrane integrity was assessed using the hypo-osmotic swelling test (HOST). Seminal smears were prepared for Giemsa staining and were assessed for morphology. All the methods were performed as in Premrov Bajuk et al. [52]. Two repeats were included in the semen analyses, except for the CASA analysis, for which three repeats were included.

The endpoints assessed with the CASA were motility, progressive motility, average path velocity (VAP), straight line velocity (VSL), curvilinear velocity (VCL), amplitude of lateral head (ALH), linearity (LIN), and elongation (ELON). The endpoints assessed in the Giemsa-stained slides for morphological abnormalities were normal spermatozoa, head abnormalities, acrosome abnormalities, neck abnormalities, midpiece abnormalities, proximal droplet, mid droplet, distal droplet, and multiple abnormalities. The endpoints assessed with the HOST test were spermatozoa that showed tail swelling or curling and spermatozoa without tail swelling or bending. For both tests, we examined 200 spermatozoa per slide with immersion oil under 1000× magnification.

2.9. Statistical Analyses

The statistical analysis was performed using the statistical software R, version 4.0.5 [53]. Differences in the above parameters between the control group and the treated group were tested separately for each variable using univariate hypothesis tests. For numerical variables (i.e., body weight, testis weight, morphometry, sperm parameters) we first used the Shapiro–Wilk test to test whether the data were normally distributed and, if necessary, applied the F-test to compare variances. If normal distribution and equal variance were not rejected, we compared the groups with the two-sample *t*-test; otherwise, the non-parametric Wilcoxon rank sum test was applied. To compare histopathological endpoints, we used Fisher's exact test. Due to multiple comparisons, we adjusted the p values with a Benjamini–Hochberg correction. A p value of less than 0.05 was considered statistically significant.

As two rams (one ram from the control group and one ram from the treated group) were deemed in a peripubertal stage, we excluded them from statistical analyses of testis and epididymis histopathology, morphometry, and sperm analysis, and presented the results for rams that were sexually mature.

3. Results

3.1. Rams' Exposure to BPA

In this study, the C_{max} of BPA was obtained between 20 min and 1 h; in only 1 animal (no. 5), the T_{max} was 6 hours. This confirms the two maxima in the time concentration curve between zero and eight hours (Figure 1). The mean C_{max} was 10.93 µg/L (CV of 17.4%). The k_{el} was calculated from the elimination part of the toxicokinetic curve, but this part started in most of the animals at six hours, after the second maximum; therefore, the accuracy of the extrapolation is low. The mean $t_{1/2}$ was 7.8 h (CV of 27.9%), calculated based on the k_{el}.

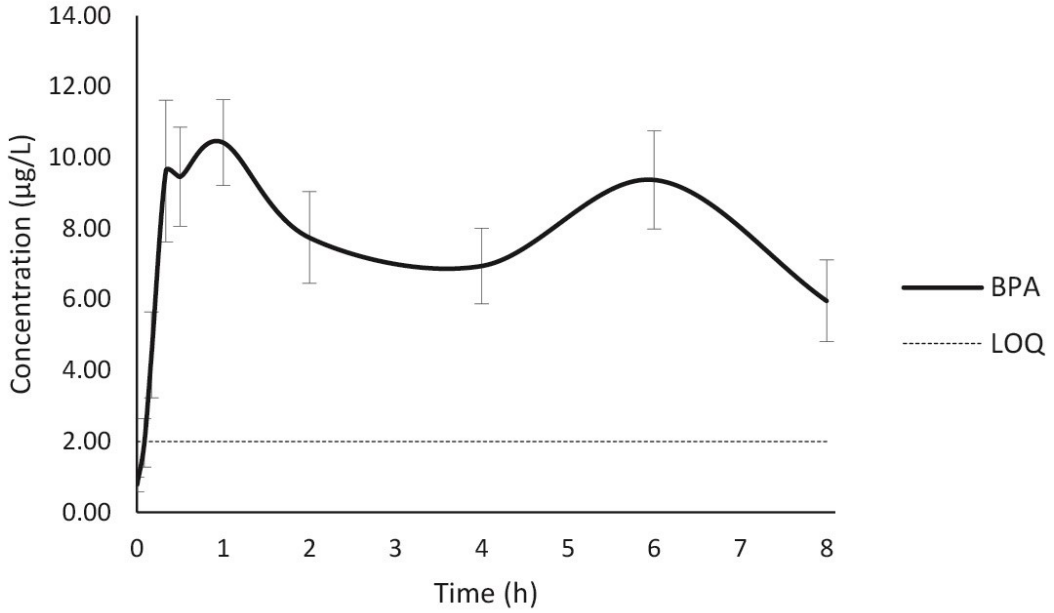

Figure 1. Time course of plasma bisphenol A (BPA) concentration with confidence interval at 95% as error bars and limit quantification (LOQ).

The AUC from zero to eight hours was 63.4 µg·h/L (CV of 17.2%), and the AUC_i based on the k_{el} was 129.4 µg · h/L (CV of 22.3%). The extrapolated part after eight hours was long due to the k_{el} uncertainty. The mean AUMC was 1569.9 µg·h^2/L (CV of 45.1%) and MRT was 11.8 h (CV of 24.4%), which represents the average time the molecule stays in the body after the first administration of the drug. The mean CL, calculated based on the dose 25 µg/kg bw, was 0.201 L/h/kg bw (CV of 21.1%), and the mean Vd was 2.2 L/kg bw (CV of 25.9%). Calculated toxicokinetic parameters are presented in the Supplementary Table S5.

3.2. Mass Measurements

No statistically significant differences were observed in the body weights and testis weights between the control and treated group (Table 1).

Table 1. Mass measurements of final body weights and testis weights of rams in the control group and the treated group exposed to 25 µg of BPA per kg of body weight per day. The mean values and standard deviations (SD) are reported.

	Control (n = 7) Mean ± SD	Treated (n = 7) Mean ± SD
Body weight (kg)	54.2 ± 7.4	52.6 ± 4.1
Testis, left (g)	150.4 ± 74.1	120.8 ± 27.2
Testis, right (g)	150.8 ± 69.3	118.4 ± 27.2

3.3. Histopathology

3.3.1. Testes

Histopathology findings in the testicular tissue in both groups were mononuclear infiltrates, multinucleated cells, segmental hypoplasia, and vacuolization of Sertoli cells. In addition, focal mineralization was found in the rete testis of a control ram. The severity of these findings was minimal, and none of these findings occurred significantly more frequently in BPA-exposed rams than in rams from the control group (Table 2). All lesions were considered normal alterations that may be encountered in control animals, and were deemed to be within the range of spontaneous alterations. The PWG characterized all findings as non-induced. Images of the most important and/or common changes are presented in Figure 2 for the testicular parenchyma and interstitial tissue, and in Figure 3 for the rete testis.

The status of the animals was considered sexually mature, except for two animals, one control animal (no. 2) and one treated animal (no. 1), which were considered to be in a peripubertal stage.

Table 2. Incidence of histopathological findings in right and left testes and rete testis of control and BPA-treated rams. Only mild lesions were detected in every endpoint examined.

	Right Testis		Left Testis		Rete Testis		
Endpoint Examined	**Control Group**	**Treated Group**	**Control Group**	**Treated Group**	**Endpoint Examined**	**Control Group**	**Treated Group**
Perivasculitis	0/6	0/6	0/6	1/6	Mineralization	1/6	0/6
Sertoli-only tubules	0/6	0/6	0/6	0/6	Multinucleated cells	1/6	0/6
Segmental hypoplasia	1/6	1/6	1/6	0/6	Mononuclear infiltrates	0/6	0/6
Vacuolation of Sertoli cells	0/6	0/6	1/6	0/6	Fibrosis	0/6	0/6
Multinucleated cells	2/6	1/6	4/6	1/6			
Mononuclear infiltrates	5/6	5/6	4/6	6/6			
Sperm retention	0/6	0/6	0/6	0/6			
Sperm head phagocytosis in the basal Sertoli cell cytoplasm	0/6	0/6	0/6	0/6			
Leydig cells atrophy	0/6	0/6	0/6	0/6			
Leydig cells hypertrophy/hyperplasia	0/6	0/6	0/6	0/6			
Leydig cell vacuolation	0/6	0/6	0/6	0/6			

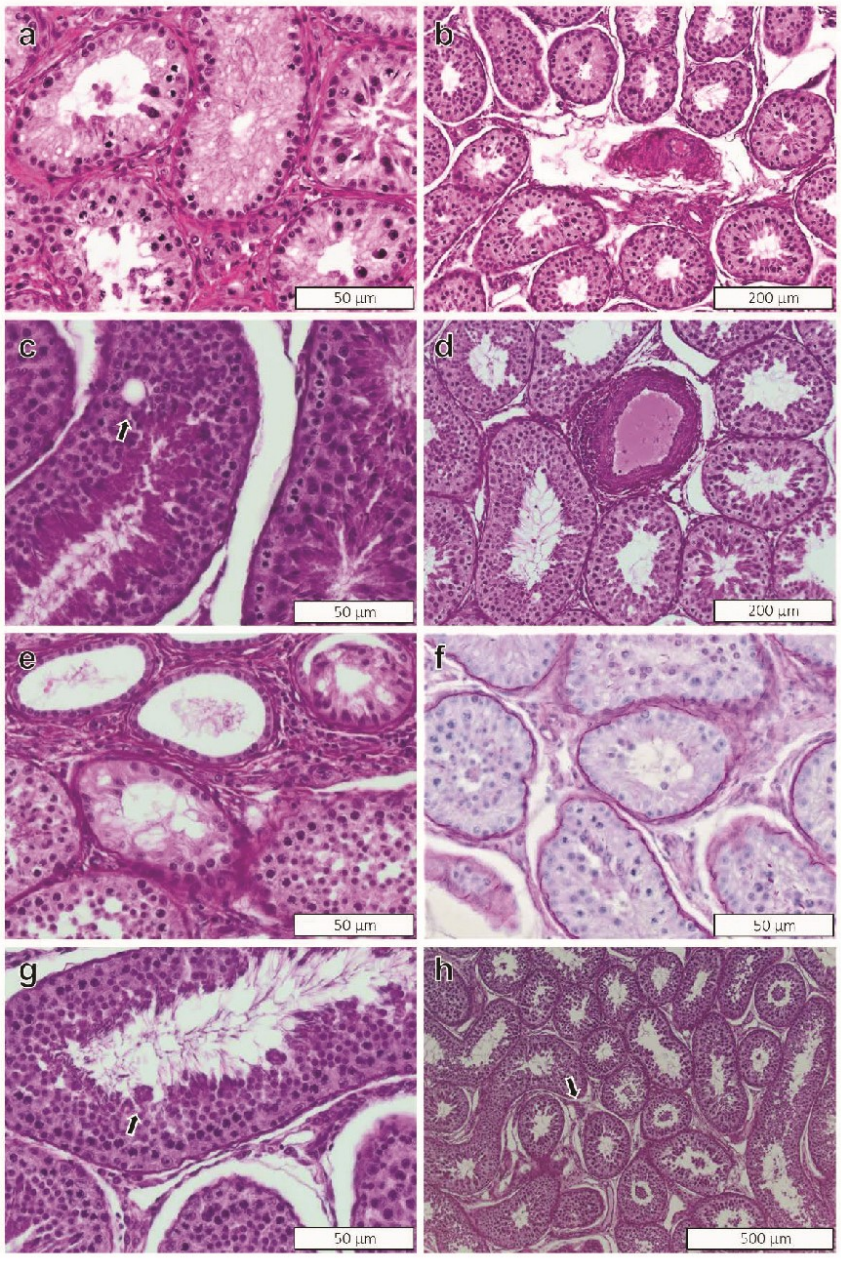

Figure 2. Testes of rams. Spontaneous changes in testis parenchyma and interstitial tissue of rams regardless of treatment group. (**a**) Testis from ram no. 2 with immature testis; (**b**) testis from ram no. 1 with partially mature testis; (**c**) vacuolation of Sertoli-only cell; (**d**) periarteritis; (**e**) hypoplastic tubules near rete testis; (**f**) Sertoli-only tubule; (**g**) multinucleated cells; (**h**) infiltrates of mononuclear cells. H&E stain, with exception of Figure 1f which is stained with PAS-H. Magnification in figures: (**a**,**c**,**e**,**f**,**g**) = ×40; (**b**,**d**) = ×20; (**h**) = ×10.

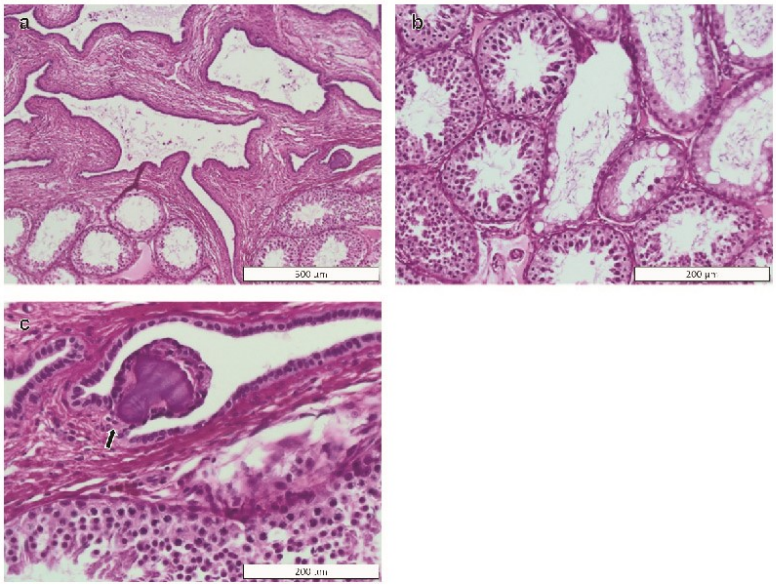

Figure 3. Rete testis of rams. Changes in rete testis of rams. (**a**,**b**) Rete testis and tubules near rete testis (segmental hypoplasia); (**c**) focal mineralization in the mesenchymal tissue of the rete testis. H&E stain. Magnification in figures: (**a**) = ×10; (**b**,**c**) = ×20.

3.3.2. Epididymides

Histopathology findings identified in the epididymides were mononuclear infiltrates, pyknotic sperm, intraepithelial fusion cysts, and sloughed epithelial cells. The severity of these findings was minimal, and with exception of pyknotic sperm that were present in BPA-treated rams, no other findings were more prevalent in BPA-exposed rams than in control rams (Table 3). The representative images of the histopathology findings in the epididymides are presented in Figure 4.

Table 3. Incidence of histopathological findings in epididymides of control and BPA-treated rams. Only mild lesions were detected in every endpoint examined.

	Endpoint Examined	Incidence of Findings in Control Rams	Incidence of Findings in Treated Rams
Head of epididymis	Mononuclear infiltrates	3/6	4/6
	Pyknotic sperm	0/6	0/6
	Sloughed epithelial cells	0/6	0/6
	Intraepithelial fusion cysts	0/6	1/6
	Sperm granuloma	0/6	0/6
Body of epididymis	Mononuclear infiltrates	4/6	5/6
	Pyknotic sperm	0/6	0/6
	Sloughed epithelial cells	0/6	0/6
	Intraepithelial fusion cysts	0/6	0/6
	Sperm granuloma	0/6	0/6
Tail of epididymis	Mononuclear infiltrates	2/6	3/6
	Pyknotic sperm	0/6	2/6
	Sloughed epithelial cells	0/6	1/6
	Intraepithelial fusion cysts	0/6	0/6
	Sperm granuloma	0/6	0/6

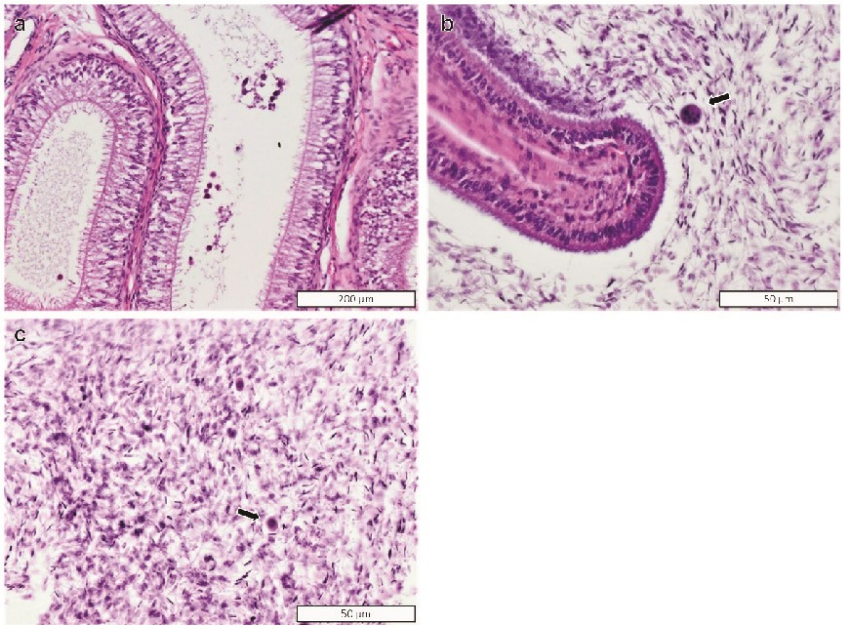

Figure 4. Epididymis of rams. (**a**) Epithelium of the head of the epididymis with cellular detritus; (**b**) pyknotic sperm; (**c**) sloughed cells. H&E stain. Magnification in figures: (**a**) = ×20; (**b**,**c**) = ×40.

3.4. Morphometric Measurements

In the exposed group, the seminiferous epithelial height was statistically significantly lower than in the control group (Wilcoxon rank sum exact test, adjusted p value = 0.0130). Although the testicular tubule diameter and area were smaller in the treated group compared to control group, there were no statistically significant differences between the two groups of rams (Table 4). Epithelial height, diameter, and area of seminiferous tubules in the testes were measured in stages VII and VIII of spermatogenesis.

Table 4. Morphometry of young adult rams exposed to 25 µg of BPA/kg bw/day compared to control rams. The mean values and standard deviations (SD) are reported.

	Control (n = 6) Mean ± SD	**Treated (n = 6) Mean ± SD**
Seminiferous epithelial height (µm)	58.44 ± 6.97	48.71 ± 2.34
Seminiferous tubule diameter (µm)	92.28 ± 9.04	89.48 ± 6.35
Seminiferous tubular area (mm^2)	26,988 ± 5301	25,255 ± 3453

3.5. Spermatozoa Analysis

There were no statistically significant differences between the control group and the treated group in any of the examined endpoints (Table 5 and Supplementary Material S5).

Table 5. Concentration, HOST, progressive motility, and normal morphology of ram spermatozoa after two months of treatment vs. non-treatment. The mean values and standard deviations (SD) are reported.

	Head of Epididymis		**Body of Epididymis**		**Tail of Epididymis**		***Ductus deferens***	
Parameter	Control (n = 6)	Treated (n = 6)	Control (n = 6)	Treated (n = 6)	Control (n = 6)	Treated (n = 6)	Control (n = 6)	Treated (n = 6)
Concentration ($\times 10^8$)	1.2 ± 0.9	0.9 ± 0.3	1.4 ± 1.6	1.1 ± 0.7	6.4 ± 1.6	4.4 ± 0.4	0.4 ± 0.4	0.3 ± 0.2
HOST (% of live sperm)	54 ± 6	56 ± 11	64 ± 9	64 ± 11	71 ± 8	67 ± 8	61 ± 7	56 ± 17
Motility (%)	4.6 ± 5.2	1.1 ± 0.6	25.7 ± 15.1	24.1 ± 22.1	96.1 ± 1.1	84.7 ± 16.4	78.7 ± 27.5	55.3 ± 28.6
Progressive motility (%)	0.2 ± 0.4	0 ± 0	2.9 ± 3.5	3.8 ± 3.7	35.1 ± 5.1	30.4 ± 14.8	29.1 ± 11.3	22 ± 16.6

4. Discussion

Our study adds to the discussion of lower-dose toxicity of BPA on male reproduction and challenges the generality of findings in rodent models, using sheep, a small ruminant, as an experimental model. In this study, we did not observe any major signs of altered reproduction in the parameters investigated in rams exposed to 25 µg BPA/kg bw/day via a dietary route, except for the significantly lower epithelial height of the seminiferous epithelium in treated rams.

The dietary route was chosen to mimic realistic exposure to BPA in humans and to minimize stress that would occur with other administration routes. In our study, we chose the dose of 25 µg BPA/kg bw, which is, in many studies, described as a low dose, including those of Consortium Linking Academic and Regulatory Insights on BPA Toxicity (CLARITY-BPA) [54]. However, controversy exists regarding the term ''low dose", and some researchers stated that this should not be called a low dose as the dose of 25 µg/kg bw corresponds to a daily uptake in humans (adults—60 kg) of 1500 µg of BPA per day [55]. Despite that, in this study, we wanted to consider the most realistic human exposure scenario, with the lowest dose that would still be measurable with our analytical method, to determine the internal exposure of rams with the purpose of detecting possible male reproductive toxicity.

Our results indicate that the dose was not high enough to detect free BPA concentrations; however, we were able to detect total BPA and demonstrate the internal exposure of rams. Interestingly, in our previous study on one Istrian Pramenka sheep [44], the C_{max} for total BPA was 43.46 µg/L, a concentration approximately four times higher than in this study, in which a four-fold-lower dose was administered to the rams, indicating a potential linear toxicokinetic BPA response at concentrations lower than 100 µg/kg. Additionally, the T_{max} for total BPA in our previous study was attained at 0.33 h, that is, in a similar timeframe to this study, where the T_{max} for six rams ranged from 20 min to 1 h.

We observed lower body weights and lower absolute testicular weights in the BPA-treated group, but the differences were not statistically significant. In rodent studies, there is a general divergence in the results of the effect of BPA on body and testicular weight. The lowest and highest doses associated with lower body weight were 0.05 mg/kg bw [26] and 960 mg/kg bw [10], respectively. The lowest and highest doses associated with lower testicular weight were 0.0002 mg/kg bw [25] and 960 mg/kg bw [10], respectively.

The histopathology of testes is acknowledged as the most sensitive endpoint for detecting testicular toxicity in animals [50]. In this study, a histopathological evaluation of testes and epididymides did not reveal any BPA-induced morphological lesions. All lesions were considered normal alterations that may be encountered in control animals, and were deemed to be within the range of spontaneous alterations. Additionally, we did not detect any difference in testes maturation between the groups. The seminiferous tubules were normally developed, with all the germ cells and all the stages of spermatogenesis present. The only exceptions were two rams, one from the control group and the other from the treated group, which were peripubertal. In these two rams, microscopic features, such as hypospermiogenesis, spermatogonial proliferation, and apoptotic and sloughed germ cells

were detected in some tubules. The lack of complete spermatogenesis in some tubules may have had an impact on our ability to critically assess the testes as per protocol; thus, these two animals were excluded from further statistical analysis of testes and epididymides histopathological endpoints, and from semen analysis. We found only one study in which comparable animal models, male goats, were exposed to BPA to evaluate male reproductive toxicity. In that study, the bucks were exposed for 120 days to a 1000-fold-higher dose of BPA than in our study, and the only BPA-elicited histopathological lesion was vacuolar degeneration of the rete testis [37]. We analyzed the microphotograph of the lesion in that publication, and our opinion is that this finding is most likely not a vacuolation of the rete testis, but more likely heterotopic fat tissue. In rodent studies, the reported results are divergent; a dose as low as 0.002 mg/BPA/kg/day [31] induced histopathological lesions, whereas in other studies a dose as high as 160 mg/BPA/kg/day did not induce histopathological lesions [10]. In the epididymides, we did not detect any histopathological lesions that were more prevalent in the BPA-exposed group. Only a few investigations on adult experimental animals provided data about lesions in the rodent epididymis, and the main lesion described was a lack of sperm in the epididymal lumina [14,26,43].

In addition to the lack of findings on spermatogenesis and the absence of testicular and epididymal toxicity, sperm morphology, motility, quality, and concentration were not significantly different in BPA-exposed rams compared to the control group in our study. Comparable to the findings of Gotardo et al. [37], in which decreased the plasma membrane integrity of spermatozoa was reported, our study also detected decreased plasma membrane integrity, but it was not statistically significant. Similar to our study, two rodent studies by Qiu et al. [29] and Liu et al. [34] reported the lack of sperm morphology defects due to the BPA treatment, in contrast to one study by Kourouma et al., in which exposure to 2, 10, and 50 mg/kg/bw for 20 days resulted in sperm morphology abnormalities, such as bent tails, coiled tails, detached heads, and double tails [15]. Most rodent studies reported a decline in sperm count [9,13,15,20,27,29,31–33,56,57]; nevertheless, in some cases, sperm count was unaffected [23]. Similar results are reported for sperm motility, which was decreased in some cases [9,13,32,57] and unchanged in others [34].

The only observed effect in our study was a statistically significantly lower seminiferous epithelium height in rams exposed to BPA. Our results are in concordance with the lower seminiferous epithelium heights in the studies of Qiu et al. [29] and Ullah et al. [11], but are in contrast with the study of Ogo et al. [16], in which epithelial height of seminiferous tubules of the testes was higher in the BPA-exposed group.

The main limitation of our study is large inter-animal variability in terms of body weights and testis weights, which could mask the subtle BPA effects in histopathology and sperm parameters. Additionally, changes associated with the developing testis in testicular tissues examined histopathologically may have masked effects on the reproductive endpoints, making it difficult to identify endocrine-disrupting effects. The second limitation of the study is the missing data of epididymis weights, as they were not measured due to complicated logistics. The third limitation of the study is that we did not test multiple BPA doses; thus, we cannot address the dose response of BPA in this study. The number of the animals and the duration of our study were limited due to the complexity of the experiment and the use of large food-producing animals. However, in our opinion, there is insufficient evidence in the rodent studies conducted to date that the dose effect is indeed non-monotonic [12,15]. In previously mentioned rodent studies investigating male reproductive toxicity of BPA, the dose response was mostly dose-dependent, and was rarely equal or non-monotonic [12,15,22,32]. Even in the two-generation CLARITY-BPA studies, no non-monotonic dose response was found. In fact, both studies concluded that rat testes and spermatozoa were insensitive to oral BPA exposure over a wide dose range (from 2.5 to 25,000 μg of BPA/kg bw) [58,59].

In conclusion, our results demonstrate that BPA did not arrest the pubertal development of the rams, and did not cause overt toxicity. Additionally, this manuscript presents the partial results of a larger study, in which dietary BPA exposure on femoral morphology,

metabolism, mineral content, and biomechanical behavior in young rams was studied [60] and in which, coincidently, no greater effects on bones were detected. Regardless of the absence of effects in this study, further studies with a larger number of animals and, perhaps, a longer duration would be required to confirm our findings.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/toxics10050224/s1, Supplementary Table S1: Birth dates of rams; Supplementary Tables S2–S4: Parameters of a basic veterinary examination and blood analysis of rams prior to the experiment; Supplementary Material S3: HPLC analysis of blood plasma samples with quality assurance procedures, validation of bisphenol A (BPA) analysis, and performance characteristics of BPA analysis [61,62]; Supplementary Table S5: Toxicokinetic parameters of rams exposed to the first dietary BPA administration; Supplementary Material S5: Results of spermatozoa analysis of rams.

Author Contributions: Conceptualization, M.P., V.C.-F., and S.Š.; methodology, V.C.-F., T.Š., K.W., P.K., E.S.-N., A.Š., A.F., M.P., V.F., Y.O., T.R., J.L., R.K., V.C.-F., and S.Š.; formal analysis, T.K.; investigation, S.Š., K.W., P.K., E.S.-N., A.F., T.K., A.Š., Y.O., T.R., J.L., R.K., V.F., T.Š., and V.C.-F.; resources, A.Š.; writing—original draft preparation, S.Š.; writing—review and editing, S.Š., V.C.-F., K.W., P.K., E.S.-N., A.F., T.K., V.F., Y.O., T.R., J.L., R.K., and T.Š.; visualization, S.Š.; supervision, V.C.-F. and T.Š.; project administration, V.C.-F.; funding acquisition, V.C.-F., M.P., and T.Š. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The animal study protocol was conducted in accordance with the Slovenian Animal Protection Act (Official Gazette of the Republic of Slovenia 38/2013), and the Council Directive 2010/63/EU, and was approved by the Ethics Committee of the Administration of the Republic of Slovenia for Food safety, Veterinary and Plant protection (protocol code U34401-3/2015/8, date of approval: 19 April 2014, and U34401-3/2015/17, date of approval 28 June 2017).

Data Availability Statement: The data presented in this study are available on request from the corresponding author.

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Conflicts of Interest: The authors declare no conflict of interest.

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3 RAZPRAVA

Preučevanje TK BPA pri ovci in ovnih

Širša javnost in raziskovalci so zadnja leta vse bolj zaskrbljeni zaradi možnega vpliva tudi zelo nizkih odmerkov KPHM, in sicer predvsem na prostoživeče živali in ljudi (162). Ena izmed bolje preučevanih snovi, ki spada med KPHM, je BPA. V literaturi ni veliko raziskav o absorpciji, distribuciji in izločanju preučevane snovi pri sesalcih, še posebej v naravnem okolju in pri koncentracijah, ki so v njem lahko prisotne. V prvem sklopu doktorske naloge smo zato preučevali pot BPA skozi organizem in njegovo izločanje v okolje pri ovci v laktaciji s sesnim jagnjetom po dietarnem in podkožnem vnosu (prva raziskava) in pri ovnih po dietarnem vnosu (druga raziskava).

Predpogoja za izvedbo TK analiz sta bila razvoj in validacija kemijskih analitskih metod v bioloških vzorcih ovce in ovnov, ki sta omogočila dokazovanje zelo nizkih koncentracij BPA v vzorcih. Glede na razpoložljivo inštrumentalno opremo smo na podlagi dostopne literature razvili metodo za HPLC-FL, s katero smo v bioloških vzorcih določali tako prosti kot celokupni BPA.

Za izolacijo celokupnega BPA smo vzorce predhodno dekonjugirali z encimom β-glukuronidaza. Pri določevanju tako prostega kot celokupnega BPA, smo vzorce krvne plazme, mleka in iztrebkov ekstrahirali z organskim topilom acetonitrilom, v katerem je BPA dobro topen in ki dobro obarja proteine in denaturira encime. Vzorce urina pa smo redčili z vodo (prosti BPA) oz. s pufrom s pH vrednostjo 4,8 (celokupni BPA) in direktno nanesli na SPE kolonice. Za čiščenje prostega BPA v urinu in iztrebkih smo izvedli dvostopenjsko SPE, najprej s polnilom Chromabond HR-X, s sferičnim, hidrofobnim kopolimerom polistirena in divinilbenzena (PS/DVB) (Macherey-Nagel, Düren, Nemčija), nato pa še z visoko selektivnim sorbentom MIP v ekstrakcijskih kolonicah AFFINIMIP SPE (AFFINISEP, Petit-Couronne, Francija). Za čiščenje prostega BPA v krvni plazmi in mleku smo zaradi manjšega biološkega ozadja uporabili le SPE z MIP sorbentom, za čiščenje celokupnega BPA pa smo pri vseh matriksih uporabili dvostopenjsko SPE, z obema zgoraj navedenima sodobnima sorbentoma. SPE smo izvedli po postopku Deceunincka in sod., ki je bil razvit za analizo prostega BPA v

živilih (163). Izpustili pa smo zadnji del njihove metode in sicer predkolonsko derivatizacijo, ki je zaradi uporabljene HPLC ni bilo potrebno izvesti, pri fluorescenčni detekciji pa smo izkoristili tudi dejstvo, da BPA izkazuje nativno fluorescenco. Pri analiznem delu smo uporabljali sistem nadzora kakovosti in se pri tem opirali na smernice za analitske metode in interpretacijo rezultatov, ki veljajo v Evropski uniji (164). Analitske metode smo validirali po naslednjih parametrih: linearnosti, izkoristku, preciznosti, meji detekcije (LOD) in meji kvantifikacije (LOQ). Linearnost ugotavljanja prostega in celokupnega BPA je bila dobra, saj je bil kvadrat korelacijskega koeficienta r^2 večji ali enak 0,99 za vse vrste matriksov. Izkoristek za prosti BPA v vzorcih krvne plazme, mleka, urina in iztrebkov se je gibal med 41,6 in 88,4 %, izkoristek za celokupni BPA pa se je gibal med 42,0 in 67,6 %. LOD za prosti BPA v krvni plazmi je bila 0,05 µg/l, v mleku 0,1 µg/l, v urinu 0,1 µg/l in iztrebkih 2 µg/kg. LOD za celokupni BPA v krvni plazmi je bila 0,4 µg/l, v mleku 0,2 µg/l, v urinu 10 µg/l in iztrebkih 1 µg/kg. LOQ za prosti BPA v krvni plazmi je bila 0,25 µg/l, v mleku in urinu 0,5 µg/l, v iztrebkih pa 2 µg/kg. LOQ za celokupni BPA v krvni plazmi je bila 1 µg/l, v mleku 0,5 µg/l, v urinu 100 µg/l in v iztrebkih 2 µg/kg.

V raziskavi, ki smo jo opravili na eni ovci s sesnim jagnjetom, smo ugotavljali vpliv različnih poti vnosa BPA na TK parametre BPA v krvni plazmi, mleku, urinu in iztrebkih ovce. Vpliv vnosa na TK parametre smo preučevali, ker smo želeli v raziskavi z ovni uporabiti najrealnejši možen vnos, ki smo mu načeloma izpostavljeni ljudje, tj. s hrano. Med raziskovalci namreč poteka razprava o tem, ali bi bilo za raziskave, ki ugotavljajo škodljiv vpliv KPHM, treba opustiti peroralni vnos s sondo (106). Vzrok za razpravo so raziskave, pri katerih so ugotovili, da se BPA lahko absorbira že v ustni votlini, ki je pri vnosu BPA po sondi izvzeta (5, 21). Absorpcija snovi pri t. i. dietarnem vnosu namreč poteka učinkovito tudi preko sluznice ustne votline, kar vodi do hitrega prenosa v arterije, ki to snov prenašajo v tkiva. Pomembno je, da se kemikalije, ki se absorbirajo po tej poti, izognejo metabolizmu prvega prehoda v črevesju in jetrih. Poleg tega je peroralni vnos po sondi povzročil stresni odziv (165) pri živalih v postopkih, kar je pri preučevanju KPHM še posebej problematično. Ker pa za tako nizke odmerke BPA (velikostnega reda 100 µg/kg t. m. BPA/dan), kot smo jih želeli uporabiti, v literaturi nismo našli podatka, ali bomo BPA v krvni plazmi ovc z uporabljeno analitsko metodo sploh lahko zaznali, smo v raziskavo vključili še podkožni vnos.

V raziskavi z ovco smo ugotovili, da je bila po dietarnem vnosu C_{max} prostega BPA v krvni plazmi 2,15 µg/l dosežena v 0,33 ure (t_{max}). AUC je bila 1,28 µg h/l. TK parametri prostega BPA po podkožnem vnosu so se razlikovali od parametrov po dietarnem vnosu, in sicer je bila C_{max} prostega BPA v krvni plazmi 6,41 µg/l dosežena v dveh urah. AUC je bila 33,3 µg h/l. Podkožni vnos je povzročil večjo in daljšo izpostavljenost prostemu BPA v primerjavi z dietarnim vnosom. Naši rezultati so podobni raziskavi Guignarda in sod., v kateri so prav tako pri ovcah primerjali dietarni in podkožni vnos (166). V naši raziskavi je bila C_{max} prostega BPA pri podkožnem vnosu trikrat višja kot pri dietarnem vnosu, v njihovi raziskavi pa je bila C_{max} prostega BPA pri podkožnem vnosu 4,6 ± 1,5-krat višja kot pri dietarnem vnosu. V raziskavi Guignarda in sod. je bila C_{max} po dietarnem vnosu dosežena zelo hitro, v 0,12 ure pri dveh ovcah in v 0,20 ure pri drugih dveh, medtem ko je bila C_{max} po podkožnem vnosu dosežena po 2 urah pri treh ovcah in po 1 uri pri eni ovci. Rezultati naše raziskave so bili podobni rezultatom njihove raziskave tudi glede TK parametrov BPAG v krvni plazmi. V primerjavi s prostim BPA je časovni potek koncentracije BPAG pri obeh načinih izpostavljenosti zelo podoben (166). Za BPAG sicer velja, da nima biološke aktivnosti (101). Guignard in sod. so izračunali, da je biološka razpoložljivost BPA lahko kar 30-krat nižja ob dietarnem vnosu v primerjavi s podkožnim vnosom (166). Glede na pridobljene rezultate lahko potrdimo del hipoteze, da način vnosa vpliva na TK profil BPA, in sicer da dietarni vnos povzroči manjšo in krajšo izpostavljenost prostemu BPA.

Izmerjene koncentracije prostega in celokupnega BPA v krvni plazmi ovce, skupaj z meritvami prostega in celokupnega BPA v mleku, so nam služile kot osnova za TK model, ki smo ga razvili za oceno izločanja BPA v ovčje mleko. S TK modelom smo ocenili delež odmerka, izločenega z mlekom, ki je bil manj kot 0,1 % tako za prosti kot za celokupni BPA ne glede na način vnosa. Ta rezultat je primerljiv z rezultati raziskave, ki so jo opravili Snyder in sod. Pri podganah so 8 ur po vnosu ugotovili le majhen delež ^{14}C-BPA (0,63 +/- 0,13 µg/equiv/mL) (114). S TK modelom smo na podlagi nižje vrednosti informacijskega kriterija Akaike (AIC) ugotovili, da se najverjetneje v mlečno žleze prenese le prosti BPA. Ker pa smo v mleku zaznali tudi celokupni BPA, ki smo ga na isti časovni točki izmerili več kot prostega BPA, domnevamo, da se v mlečni žlezi prosti BPA metabolizira v BPAG. Prav tako so Doerge in sod. pri glodavcih posredno dokazali, da se v mlečno žlezo v večji meri prenese prosti BPA, v manjši meri pa BPAG (113). Domnevamo, da bi se lahko prosti BPA v mlečni žlezi v BPAG metaboliziral s

pomočjo encima uridin 5′-difosfo-glukuronoziltransferaze (UDP-glukuronoziltransferaze). V posameznih raziskavah so v tkivu dojk žensk namreč ugotovili prisotnost encimov UDP-glukuronoziltransferaz (UGT) (167-169). Rezultati raziskave Streetove in sod. pa potrjujejo sposobnost glukuronidacije BPA v tkivu dojk pri ženskah, čeprav so aktivnosti glukuronidacije veliko nižje (za več kot 100000-krat) v primerjavi s tistimi, ki so bile ugotovljene v jetrih (170). V literaturi nismo zasledili raziskav, ki bi ugotavljale prisotnost UGT v mlečni žlezi ovc, domnevamo pa, da so mlečne žleze vseh sesalcev verjetno opremljene s primerljivimi mehanizmi razstrupljanja.

V vzorcu mleka ovce smo po dietarnem vnosu ocenili prisotnost prostega BPA na 0,05 µg/l in izmerili 0,78 µg/l celokupnega BPA. Po podkožnem vnosu smo v vzorcu mleka ovce izmerili 0,87 µg/l prostega BPA in 1,89 µg/l celokupnega BPA. Prvo hipotezo, da je v vzorcih mleka ovce mogoče zaznati BPA po dietarnem in podkožnem vnosu, smo tako potrdili.

V prvi raziskavi smo ovci odvzeli tudi vzorce urina in iztrebkov. Ker nismo imeli na razpolago metaboličnih kletk, nismo mogli zajeti vsega urina in iztrebkov, zato deleža vnesenega odmerka BPA v teh biološkim materialih nismo mogli izračunati. Smo pa na podlagi zaporednih meritev po vnosu BPA kljub temu ugotovili, da se je BPA, ne glede na način vnosa, večinoma izločil z urinom kot BPAG, le majhen del pa se ga je izločil kot prosti BPA. V eni časovni točki, tj. štiri ure po prvem dietarnem vnosu, smo v vzorcih urina ovce izmerili 15,81 µg/l prostega BPA in 15157 µg/l celokupnega BPA, v vzorcih iztrebkov pa sta bila prosti in celokupni BPA manjša od LOD (< 1 µg/kg). Podobno smo 4–4,5 ure po prvem podkožnem vnosu v vzorcih urina ovce izmerili 11,53 µg/l prostega BPA in 13846 µg/l celokupnega BPA, v vzorcih iztrebkov pa sta bila prosti in celokupni BPA prav tako manjša od LOD (< 1 µg/kg). Da se BPA večinoma izloča skozi ledvice v obliki BPAG, so ugotovili tudi v drugih raziskavah, opravljenih na ovcah (171, 172), opicah (92), in prašičih (173). Pri podganah je bil delež BPAG, izločenega z urinom, zgolj 6,5 % (97) zaradi enterohepatične poti BPA, ki se pri tej živalski vrsti večinoma izloča z iztrebki.

Omejitev naše raziskave je bila posredno določanje BPAG z metodo encimske dekonjugacije. Posledično je bila določena koncentracija BPAG v vzorcih urina v naši raziskavi najverjetneje višja od realne koncentracije v vzorcih, saj smo dekonjugacijo opravili z encimom ß-glukuronidaza iz *Helix pomatia* vrste HP-2, ki ima tako glukuronidazno (≥ 100.000 U/ml) kot sulfatazno (≤ 7.500 U/ml) aktivnost. To so dokazali tudi Lacroix in sod., saj je bila

koncentracija neposredno izmerjenega BPAG v njihovi raziskavi nižja od koncentracije BPAG, pridobljene z encimsko dekonjugacijo (171). *In vivo* raziskave, v katerih so določali vsebnost BPA v iztrebkih, so bile opravljene le na opicah (92) in podganah (97). Pri opicah se je BPA v veliko manjšem deležu izločil z iztrebki (1,8–3,1 %) kot pri podganah (78–82 %). Pri obeh vrstah živali so v vzorcih iztrebkov zaznali le prosti BPA (92, 97). V naši raziskavi so bile ugotovljene koncentracije prostega BPA v iztrebkih podobno nizke kot pri opicah. Prav tako smo v iztrebkih zaznali primerljivo količino celokupnega BPA in prostega BPA, kar posredno nakazuje na to, da je delež BPAG, ki se izloči z iztrebki, tudi pri ovcah zelo majhen.
Izločki živali (gnojnica in gnoj), namenjenih za proizvodnjo živil, se v nekaterih kmetijskih praksah še vedno odlagajo neposredno na kmetijske površine. Ker smo v naši raziskavi ugotovili, da se BPA pri ovcah izloča pretežno z urinom, bi uporaba gnojnice (sestavljene iz urina in iztrebkov) lahko negativno vplivala na tla in življenje v tleh. V večini raziskav je sicer koncentracija brez opaznega učinka (angl. *no-observed-effect concentration*; NOEC) za kopenske nevretenčarje visoka; za kolobarnike (*Enchytraeus crypticus*) je ≥100 mg/kg suhe mase, za skakače (*Folsomia candida*) pa je ≥ 500 mg/kg suhe mase (174). Vendar pa so raziskave z nižjimi odmerki BPA pri kopenskih nevretenčarjih še zelo redke. Ena izmed njih je raziskava Babićeve in sod., v kateri so dokazali, da že izpostavljenost koncentraciji 0,023 µg/l BPA pri deževnikih (*Eisenia fetida*) povzroča degenerativne spremembe v celični strukturi jajčnikov (175).

V raziskavi, ki smo jo opravili na ovnih, smo uporabili štirikrat nižji odmerek (25 µg/kg t. m./dan) kot v raziskavi z ovco. Pričakovali smo, da bomo glede na rezultate prve raziskave z našo analitsko metodo in koncentriranjem vzorcev lahko zaznali tudi štirikrat nižje vrednosti BPA v krvni plazmi. Žal so bile ugotovljene koncentracije prostega BPA prenizke, da bi lahko ocenili izpostavljenost ovnov tej obliki BPA. Kljub temu da prostega BPA nismo izmerili, pa so bile izmerjene koncentracije celokupnega BPA dovolj visoke, da smo na njihovi podlagi lahko naredili osnovno neprostorsko TK analizo. Ker smo v prvi raziskavi na ovci v laktaciji uporabili enako metodologijo in živalsko vrsto, vendar štirikrat višji odmerek, je rezultate zanimivo primerjati z našo drugo raziskavo z ovni. C_{max} za celokupni BPA je v raziskavi z ovco znašala 43,46 µg/l, kar je približno štirikrat višja povprečna C_{max} kot v raziskavi z ovni (10,93 +/- 1,9 µg/l). Prav tako je bil t_{max} v prvi raziskavi pri ovci dosežen v 0,33 ure, v drugi raziskavi pri šestih ovnih med 0,33–1 uro, zgolj pri enem ovnu pa šele po šestih urah. Pri vseh ovnih smo

ugotovili drugi koncentracijski vrh približno po šestih urah po dietarnem vnosu BPA. Drugega koncentracijskega vrha pa nismo zaznali v prvi raziskavi z ovco v laktaciji. Tudi Guignard in sod. niso zaznali drugega koncentracijskega vrha (5, 166). Do pojava drugega koncentracijskega vrha celokupnega BPA po šestih urah bi po naših ugotovitvah lahko prišlo zaradi potencialno prisotne enterohepatične cirkulacije.

Drugega dela druge hipoteze, ki se nanaša na vpliv fiziološkega obdobja živali, tj. vpliva starosti in spola živali na TK profil BPA nismo morali ne potrditi, ne zavreči, saj so bile vrednosti prostega BPA v krvni plazmi ovnov prenizke, da bi jih lahko uporabili v TK analizi in posledično medsebojna primerjava TK profilov ovce in ovnov ni bila mogoča.

Preučevanje vpliva BPA na spolne organe ovnov v puberteti

Namen drugega sklopa doktorske naloge je bil pridobiti podatke o vplivu BPA na spolne organe ovnov in s tem ugotoviti vpliv na njihovo reproduktivno sposobnost. Rezultati naše raziskave, v kateri so bili ovni izpostavljeni 25 µg//kg t. m. BPA/dan po prehranski poti in pri katerih smo ob koncu dvomesečne izpostavljenosti ugotavljali vpliv BPA na telesno maso, absolutno maso mod, vpliv na histopatologijo mod in nadmodkov, nekatere morfometrične značilnosti zvitih semenskih cevk in vpliv na kvaliteto ter kvantiteto semena, prispevajo k boljšemu poznavanju toksičnosti nizkih odmerkov BPA na moško reprodukcijo.

Prva posebnost naše druge raziskave je bila uporaba eksperimentalnega živalskega modela, ki v primerjavi z dostopnimi podatki v znanstveni literaturi niso bile miške ali podgane, druga posebnost je bila uporaba dietarnega vnosa, tretja posebnost pa je bila uporaba relativno nizkega odmerka BPA, in sicer tako nizkega, da po mnenju nekaterih raziskovalcev predstavlja realen vnos BPA za ljudi (5, 116).

Ovni so bili v raziskavi izbrani iz dveh razlogov. Eden je bil ugotavljanje, kako splošne so ugotovitve rezultatov raziskav, opravljenih na glodavcih, drugi pa njihova velikost, ki nam je omogočila zadostno količino odvzetih bioloških materialov, da smo lahko potrdili notranjo izpostavljenost BPA.

Odmerek 25 µg//kg t. m. BPA/dan smo v raziskavi izbrali, ker je v številnih raziskavah, vključno z nedavno izvedenimi raziskavami konzorcija, ki povezuje akademska in regulativna

spoznanja o toksičnosti BPA (angl. *Consortium Linking Academic and Regulatory Insights on BPA Toxicity;* CLARITY-BPA), opisan kot nizek odmerek (176). Poleg tega pa smo na podlagi predhodne raziskave na eni ovci in opcijsko večjim koncentriranjem vzorcev krvi za inštrumentalno analizo presodili, da bomo po tem odmerku lahko dokazali notranjo izpostavljenost prostemu BPA. Odmerek 25 µg/kg telesne mase pri povprečnem odraslem človeku s 60 kg telesne mase pomeni izpostavljenost 1,5 mg BPA na dan, česar nekateri raziskovalci sicer nimajo za nizko izpostavljenost BPA (177).

V naši raziskavi smo pri ovnih, izpostavljenih BPA, ugotovili nižjo telesno maso in maso mod v primerjavi z ovni iz kontrolne skupine, vendar pa razlika ni bila statistično značilna. V preostalih raziskavah, v katerih so preučevali vpliv BPA na moške spolne organe glodavcev v puberteti ali v odraslem obdobju, so bili rezultati zelo različni. Tako je bil najnižji odmerek, ki je povzročil nižjo telesno maso mišk/podgan, 0,05 mg/kg t. m./dan (140), najvišji odmerek pa 960 mg/kg t. m./dan (136). Najnižji in najvišji odmerek, povezan z manjšo maso mod, je bil 0,0002 mg/kg t. m./dan (139) oziroma 960 mg/kg t. m./dan (136).

V literaturi za najobčutljivejšo preiskavo, s katero se dokazuje toksičnost za moško reprodukcijo pri živalih v postopkih, velja histopatološka preiskava mod in nadmodkov (178). V naši raziskavi s to preiskavo nismo ugotovili morfoloških sprememb, ki so značilne za KPHM in ki bi se pri ovnih, izpostavljenih BPA, pojavljale pogosteje kot pri ovnih iz kontrolne skupine. Histopatološko preiskavo mod z upoštevanjem faz spermatogeneze (179-181) smo opravili po priporočilih za oceno mod in nadmodkov na reproduktivno toksičnost (178, 182). Ugotovili smo, da so bile zvite semenske cevke normalno razvite in so imele vse zarodne celice in ohranjene stopnje spermatogeneze. Izjema sta bila dva ovna – eden iz kontrolne skupine in drugi iz tretirane skupine, ki sta bila ob koncu postopka v obdobju peripubertete. To je bil tudi razlog, da smo ju izključili iz statistične analize rezultatov. V literaturi smo zasledili le eno raziskavo, v kateri so uporabili primerljivo živalsko vrsto. V tej raziskavi so bili odrasli kozli 120 dni izpostavljeni 1000-krat večjemu odmerku BPA kot ovni v naši raziskavi, edina histopatološka sprememba, ki jo je povzročil BPA, pa je bila vakuolarna degeneracija rete testisa (161). Glede na fotografijo, ki prikazuje opisane spremembe, menimo, da je prišlo do napačne interpretacije pri histopatološki preiskavi in da v resnici ne gre za vakuolizacijo rete testisa, temveč za heterotopno maščobno tkivo v vezivu rete testisa, in da histopatoloških

sprememb, ki bi nakazovale na toksičnost BPA, v tej raziskavi niso ugotovili. V raziskavah na glodavcih, ki so bili BPA izpostavljeni v času pubertete ali v odraslem obdobju, so rezultati zelo različni; Jin in sod. so ugotovili zmanjšanje števila spermatogonijev, spermatocit in spermatid že pri odmerku 0,002 mg BPA/kg t. m./dan (149), medtem ko Li in sod. po odmerku 160 mg BPA/kg t. m./dan niso ugotovili histopatoloških sprememb (136). V raziskavah konzorcija CLARITY-BPA pri podganah, ki so bile izpostavljene BPA v odmerkih 2, 5, 25, 250, 2500 in 25000 µg/kg t. m., niso ugotovili histopatoloških sprememb, za katere bi lahko trdili, da jih je povzročil BPA, kljub temu da so bile podgane BPA izpostavljene že od 6. dneva brejosti pa do 21. dne po rojstvu ali do konca postopka, tj. eno oz. dve leti (9, 183). Zanimivo je, da v večini *in vivo* raziskav na glodavcih, pri katerih so ugotavljali reproduktivno toksičnost, histopatološka preiskava sploh ni bila izvedena (135, 152, 156, 158, 184-186), lahko pa je bila izvedena, vendar niso bile upoštevane smernice za določanje histopatoloških sprememb za ugotavljanje toksičnosti (136, 143, 147, 150, 151). V naši raziskavi niti v modih niti v tkivu nadmodkov nismo ugotovili sprememb, ki bi nakazovale na toksično delovanje BPA na moda ali nadmodke. Kot v modih smo tudi v nadmodkih sicer v obeh skupinah ugotovili minimalne spremembe, vendar nobena izmed teh ni bila pogosteje prisotna pri ovnih, izpostavljenih BPA. Kljub temu da v priporočilih za oceno mod in nadmodkov na reproduktivno toksičnost izpostavljajo pomembnost histopatološke preiskave nadmodkov, so v raziskavah na glodavcih le redko poročali o rezultatih te preiskave. Le v treh raziskavah na odraslih glodavcih so poročali o spremembah v nadmodkih, glavna opisana sprememba pa je bila pomanjkanje semenčic v lumnih kanalčkov nadmodkov (140, 152).

Poleg histopatološke preiskave mod in nadmodkov smo na zvitih semenskih cevkah mod opravili tudi morfometrične meritve premera cevk, površine cevk in višine zarodnega epitela v zvitih semenskih cevkah. Presenetljivo je bila višina zarodnega epitela v zvitih semenskih cevkah nižja pri ovnih, ki so bili izpostavljeni BPA. Razlog za opisano razliko bi bil lahko manjše število zarodnih celic v zvitih semenskih cevkah.

V naši raziskavi morfologija, gibljivost, parametri smeri in hitrosti gibanja semenčic ter integriteto celične membrane semenčic med skupinama ovnov niso bili statistično značilno različni. Rezultate naše raziskave zaradi razlik v metodologiji težko primerjamo z rezultati raziskav na glodavcih. V naši raziskavi smo namreč nadmodke zaradi njihove velikosti razdelili

na posamezne dele (glava, telo, rep nadmodka) in analize semena izvedli na vsakem delu nadmodka in na semenovodu. Pri glodavcih, kjer je nadmodek manjši, je vsak nadmodek predstavljal le en vzorec. Sicer so rezultati raziskav na glodavcih o vplivu BPA na seme zelo različni. Podobno kot v naši raziskavi sta dve raziskavi na glodavcih, ki so ju izvedli Qiu in sod. (145) ter Liu in sod. (155), poročali o odsotnosti morfoloških napak semenčic. Variabilni so bili tudi rezultati raziskav, v katerih so ugotavljali spremembo gibanja semenčic. V nekaterih raziskavah se je gibljivost zmanjšala (135, 151, 157, 186), v drugih pa se ni spremenila (155). V primerjavi z raziskavami na glodavcih, v katerih so večinoma poročali o zmanjšanju števila semenčic (135, 141, 145, 149, 151, 154, 157, 158, 184, 186, 187), pa v naši raziskavi BPA ni statistično značilno vplival na koncentracijo semenčic v posameznem delu nadmodka ali v semenovodu.

Na podlagi vseh rezultatov smo tretjo hipotezo, da dvomesečna izpostavljenost BPA s hrano v odmerku 25 µg/kg telesne mase/dan vpliva na morfološke značilnosti mod in osnovne parametre semena ovnov, zavrnili. Ker smo ugotovili nekatere razlike, ki bi lahko nakazovale na škodljive učinke BPA, ki z izjemo nižjega zarodnega epitela zvitih semenskih cevk niso bile statistično značilne, menimo, da bi bila za zanesljivo potrditev naših rezultatov potrebna raziskava z večjim številom živali v postopku. Kljub temu pa smo v toksikoloških raziskavah večinoma zasledili, da pri večjih živalskih vrstah uporabljajo zgolj štiri živali istega spola na skupino (188). To bi lahko pomenilo, da so štiri živali za relevantnost rezultatov postopka dovolj, lahko pa tudi, da rezultati takih preiskav niso zanesljivi.

4 SKLEPI

- V vzorcih mleka ovce, izpostavljene BPA, smo po dietarnem in podkožnem vnosu zaznali prosti in celokupni BPA. Na podlagi tega menimo, da je prenos BPA v mleko pri prežvekovalcih možen, a je minimalen. Poleg tega smo na podlagi TK modela ugotovili, da BPA v mlečno žlezo ovce v večji meri prehaja v prosti, nekonjugirani obliki. Glede na to ugotovitev in glede na izmerjene vrednosti BPA-konjugata in celokupnega BPA v mleku domnevamo, da se prosti BPA verjetno naknadno presnovi v mlečni žlezi.
- TK parametri po dietarnem in podkožnem vnosu BPA so različni. Dietarni vnos je v naši raziskavi povzročil manjšo in krajšo izpostavljenost prostemu BPA, medtem ko način vnosa ni vplival na TK parametre celokupnega BPA.
- V tkivu mod in nadmodkov nismo ugotovili histopatoloških sprememb, ki bi nakazovale, da BPA deluje toksično. BPA pa bi lahko vplival na koncentracijo semenčic v nadmodku in semenovodu, vendar razlike v naši raziskavi niso bile statistično značilne in bi bile za njihovo potrditev potrebne dodatne raziskave z večjim številom živali. Edina statistično značilna razlika med skupinama je bila nižja višina zarodnega epitela zvitih semenskih cevk pri ovnih, izpostavljenih BPA.

5 POVZETEK

BPA je kemična snov, ki se globalno proizvaja in uporablja v velikih količinah. Zaradi široke uporabe je v okolju zelo razširjen. Dokazan je bil v atmosferi, vodi in odplakah, pitni vodi, hrani in celo v stanovanjskem prahu. Kljub temu da ima kratko razpolovno dobo, je v okolju stalno prisoten zaradi neprekinjenega vnosa. V organizem ljudi in živali se najpogosteje vnese s hrano in pijačo, v manjši meri tudi skozi kožo ali z vdihavanjem. Zaradi šibke estrogenske aktivnosti in sposobnosti vezave na številne druge receptorje je poznavanje njegovih TK lastnosti zelo pomembno.

Doktorsko disertacijo smo izvedli na domačih ovcah (*Ovis aries*). V prvem sklopu naloge smo preučevali TK parametre pri ovci in ovnih pasme istrska pramenka po zaporednem dietarnem in podkožnem vnosu BPA. Namen tega sklopa je bil preučiti absorpcijo in eliminacijo BPA in ovrednotiti vpliv vnosa na TK parametre BPA. Razvili in validirali smo kemijske analitske metode za določanje prostega in celokupnega BPA v kompleksnih bioloških vzorcih – v krvni plazmi, mleku, urinu in iztrebkih. Za določanje BPA smo vzorce očistili in ekstrahirali s pomočjo sodobnega sorbenta MIP, ki je selektivno vezal preiskovani analit. Za določanje celokupnega BPA oz. posredno BPAG smo vzorce pred tem dodatno dekonjugirali z encimom ß-glukuronidaza in uporabili še dodatno SPE z nosilcem Chromabond HR-X. Prav tako smo ta nosilec uporabili za osnovno čiščenje urina in iztrebkov. Uvedene metode smo validirali po osnovnih parametrih, ki so ustrezni glede na smernice za analitske metode in interpretacijo rezultatov v EU, zato menimo, da je razvoj metod uspešen. Rezultati neprostorske TK analize so pokazali, da sta TK profila BPA v krvni plazmi ovce v laktaciji s sesnim jagnjetom različna. Dietarni vnos BPA je povzročil manjšo in krajšo izpostavljenost prostemu BPA, AUC je bila 1,28 µg h/l, v primerjavi s podkožnim vnosom, pri katerem je bila AUC prostega BPA 33,3 µg h/l. TK profila celokupnega BPA oz. BPAG pa sta si bila zelo podobna. V vzorcih mleka smo po šestih urah od dietarnega vnosa ocenili prisotnost prostega BPA na 0,05 µg/l in izmerili 0,78 µg/l celokupnega BPA, po podkožnem vnosu pa smo izmerili 0,87 µg/l prostega BPA in 1,89 µg/l celokupnega BPA. S tem smo potrdili, da se BPA izloča v mleko prežvekovalcev. Poleg tega smo na podlagi meritev prostega in celokupnega BPA v krvni plazmi in mleku razvili prostorski TK model, s katerim smo ocenili izločanje BPA v mleko ovce. Delež izločenega BPA v mleko je bil ne glede na način vnosa manjši od 0,1 % prejetega vnosa. S prostorskim TK modelom smo ugotovili, da se BPA najverjetneje izloča v mleko

predvsem v prosti, nekonjugirani obliki. V urinu ovce smo po dietarnem vnosu izmerili zelo nizke vrednosti prostega BPA (≤ 16,55 µg/l) in visoke vrednosti celokupnega BPA (≤ 15157 µg/l), katerega največji del je bil najverjetneje BPAG. V iztrebkih ovce pa smo izmerili zelo nizke vrednosti prostega BPA (≤ 45,25 µg/kg) in celokupnega BPA (≤ 53,4 µg/kg). Po štirikrat manjšem odmerku, ki so ga prejemali ovni v drugi raziskavi, smo v krvni plazmi določili približno štirikrat nižjo C_{max} celokupnega BPA.

V drugem sklopu doktorske naloge smo ugotavljali vpliv BPA na spolne organe ovnov, ki so BPA prejemali v puberteti. Med skupinama nismo ugotovili statistično značilnih razlik v pojavnosti in intenziteti histopatoloških sprememb v modih in nadmodkih. Prav tako nismo ugotovili statistično značilnih razlik v premeru in površini zvitih semenskih cevk. Ugotovili pa smo statistično značilno nižjo višino zarodnega epitela zvitih semenskih cevk pri tretiranih ovnih. Telesna masa in masa mod sta bili povprečno malo nižji v tretirani skupini ovnov, vendar razlike med skupinama niso bile statistično značilne. Na semenu smo opravili osnovne teste – pogledali smo morfologijo semenčic, koncentracijo semena, integriteto celične membrane semenčic in s sistemom CASA določili hitrost in smer gibanja semenčic. Statistično značilnih razlik med skupinama nismo ugotovili. Glede na rezultate lahko zaključimo, da BPA ni (bistveno) vplival na spolne organe ovnov. Za potrditev odsotnosti vpliva BPA na spolne organe ovnov pa bi bile potrebne nadaljnje raziskave z večjim številom živali.

6 SUMMARY

BPA is a chemical that is produced and used globally in large quantities. Due to its widespread use, it is very common in the environment. It has been detected in the atmosphere, water and sewage, drinking water, food, and even in house dust. Although it has a short half-life, it is constantly present in the environment due to its continuous release. Humans and animals most commonly ingest BPA through food and beverages, and are to a lesser extent exposed to BPA through the skin or by inhalation. Since it has weak estrogenic activity and the ability to bind to a number of receptors, it is important to know its TK properties.

This doctoral dissertation was prepared on domestic sheep (*Ovis aries*). In the first part, TK parameters were studied in a ewe and rams of the Istrian pramenka breed after sequential dietary and subcutaneous administration of BPA. The aim was to study the absorption and elimination of BPA and to evaluate the influence of the administration route on the TK parameters of BPA. For the purpose of studying the TK parameters, chemical analytical methods for the determination of free and total BPA in complex biological samples - blood plasma, milk, urine and feces - were developed and validated. For the determination of BPA, samples were cleaned and extracted using an MIP sorbent that selectively bound the analyte of interest. For the determination of total BPA or indirectly BPAG, the samples were further deconjugated with ß-glucuronidase enzyme, and an additional SPE with Chromabond HR-X carrier was carried out. The latter carrier was also used for basic cleaning of urine and feces. The methods were validated against basic parameters that are appropriate according to the EU guidelines for analytical methods and interpretation of results. We therefore consider the development of the methods to be successful. The results of noncompartmental TK analysis showed that the TK profiles of BPA in blood plasma of the lactating ewe with a suckling lamb varied. Dietary administration of BPA resulted in a lower and shorter exposure to free BPA – AUC was 1.28 µg h/L, compared with subcutaneous administration, where the AUC of free BPA was 33.3 µg h/L. However, the TK profiles of total BPA and BPAG were very similar. In milk samples, 0.05 µg/l of free BPA was estimated and 0.78 µg/l of free BPA was measured six hours after dietary and subcutaneous administration. At the same sampling point after dietary and subcutaneous administration 0.87 µg/l and 1.89 µg/l of total BPA were measured in milk samples, respectively. These results confirm that BPA is excreted into ruminant milk. In addition, based on the measurements of free and total BPA in blood plasma and milk, we developed a

compartmental TK model to estimate the excretion of BPA into sheep milk. The percentage of BPA excreted in milk was less than 0.1% of the administered dose, regardless of the route of administration. Using a compartmental TK model, we estimated that BPA was most likely excreted in the free, unconjugated form. Very low levels of free BPA (≤ 16.55 µg/L) and high levels of total BPA (≤ 15,157 µg/L) were measured in the urine of sheep after dietary intake, of which the largest fraction was most likely BPAG. However, very low levels of free (≤ 45.25 µg/kg) and total (≤ 53.4 µg/kg) BPA were measured in sheep feces. After a fourfold reduction of the dose, which was received by rams in the second study, we observed an approximately fourfold reduction in the C_{max} of total BPA in blood plasma.

In the second part of the doctoral dissertation we investigated the effect of BPA on the reproductive organs of rams exposed to BPA during puberty. We found no statistically significant differences between the two groups in the incidence and intensity of histopathological changes in the testes and epididymis. We also found no statistically significant differences in the diameter and surface area of the seminiferous tubules. We did find a statistically significant lower height of the germinal epithelium of the seminiferous tubules in the treated rams. Body weight and testicular weight were on average slightly lower in the treated group of rams, but differences between the groups were not statistically significant. Basic tests were performed on semen – we analyzed the morphology of the spermatozoa, semen concentration, integrity of the spermatozoal cell membrane, and determined movement speed and direction of spermatozoa using CASA. No statistically significant differences were found between the two groups. According to the results, it can be concluded that BPA did not (significantly) affect the reproductive organs of rams. However, further studies with a larger number of animals would be needed to confirm the absence of an effect of BPA on the reproductive organs of rams.

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9 PRILOGE

PRILOGA 1: Dopolnilno gradivo prve objave z naslovom Preliminarna toksikokinetična raziskava pri mlečni ovci v laktaciji po dietarnem in podkožnem vnosu bisfenola A

Supplementary information

Preliminary toxicokinetic study of BPA in lactating dairy sheep after repeated dietary and subcutaneous administration

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Supplementary Table S1

Clinical examination of the sheep				
Date	Body temperature	Pulse rate (per minute)	Breathing frequency (per minute)	Rumination frequency (per five minutes)
20.3.2017 (1st day of the first period of the experiment)	38.7 °C	65	16	10
7.4.2017 (1st day of the second period of the experiment)	38.8 °C	72	20	10

Supplementary Table S2

Haematology			
Parameter	Value	Date of blood sampling	Date of performed analysis
Erythrocytes (x10^{12}/L)	8.80	20.3.2017	21.3.2017
Hb (g/dL)	9.6		
MCV (fL)	37		
Ht (%)	32.2		
Leukocytes (x10^{9}/L)	8.1		
MCH (pg)	10.9		
MCHC (g/dL)	29.7		
Platelets (x10^{9}/L)	509		

Neutrophils (%)	31
Eosinophils (%)	1
Basophils (%)	0
Lymphocytes (%)	68
Segmented neutrophils (%)	0
Monocytes (%)	0

Haematological analyses were performed utilising the Scil Vet abc Plus (Horiba, Japan) automated haematological analyser. Differential white blood cell count was determined according to the standard procedure; smears were stained with Hemacolor (Merck, Darmstadt, Germany) and manually counted via microscopic examination.

Supplementary Table S3

Biochemistry

Parameter	Value	Date of blood sampling	Date of performed analysis
AST (U/L)	171	20.3.2017	21.3.2017
GGT (U/L)	42		
iP (mmol/L)	2.16		
Ca (mmol/L)	2.54		
Mg (mmol/L)	1.02		
Na (mmol/L)	152		
K (mmol/L)	4.51		
Cl (mmol/L)	109		
Gluc (mmol/L)	3.0		
Urea (mmol/L)	4.80		
Crea (µmol/L)	62		
Fe (µmol/L)	24.9		

For biochemistry analysis, biochemistry analyser RX Daytona (Randox Laboratories Ltd, Crumlin; UK) was utilized.

PRILOGA 2: Dopolnilno gradivo tretje objave z naslovom Bazična raziskava dietarnega vnosa bisfenola A (BPA) ovnom pasme istrska pramenka in ugotavljanje reproduktivne toksičnosti

toxics

MDPI

Supplementary Materials: Basic Exploratory Study of Bisphenol A (BPA) Dietary Administration to Istrian Pramenka Rams and Male Toxicity Investigation

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S1. Birth Dates of Rams

Table S1. Birth dates of rams included in the experiment.

	Ram Number	Birth Date
Control group		
	10	February 12.
	9	January 27.
	13	January 19.
	2	January 27.
	3	January 29.
	6	January 26.
	14	January 30.
Treated group		
	4	January 27.
	1	January 27.
	7	January 26.
	12	January 30.
	8	January 29.
	5	January 23.
	11	January 27.

S2. Parameters of a Basic Veterinary Examination and Blood Analysis of Rams Prior to the Experiment

Table S2. Results of clinical examination of the rams prior to the experiment.

	Ram Number	Body Temperature (°C)	Pulse Rate (/min)	Breathing Frequency (/min)	Rumination Frequency (/5 min)
Control group					
	10	40.4	68	40	2
	9	39.4	68	32	2
	13	39.6	68	32	2
	2	39.8	68	24	2
	3	39.9	68	32	2
	6	39.6	68	36	2
	14	40.4	68	36	2
Treated group					

4	39.9	68	39.9	2
1	39.9	68	39.9	2
7	39.7	68	39.7	2
12	39.3	68	39.3	2
8	39.8	68	39.8	2
5	39.9	68	39.9	2
11	39.5	68	39.5	2

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Table S3. Haematology of the rams prior to the experiment.

	Ram number	Erythrocytes (x 10^{12}/L)	Hb (g/dL)	MCV (fL)	Ht (%)	Leukocytes (x 10^9/L)	MCH (pg)	MCHC (g/dL)	Platelets (x 10^9/L)	Neutrophils (%)	Eosinophils (%)	Basophils (%)	Lymphocytes (%)	Segmented neutrophils (%)	Monocytes (%)
Control group															
	10	12.06	12.1	30	35.9	6.6	10.0	33.6	417	40	0	0	59	0	1
	9	11.32	11.5	29	33.4	7.0	10.2	34.5	455	32	0	0	68	0	0
	13	11.80	12.7	30	34.8	10.0	10.7	36.4	504	23	1	0	75	1	0
	2	13.20	13.2	30	40.2	4.7	10.0	32.9	698	48	1	0	49	0	2
	3	11.86	12.0	30	36.1	9.3	10.1	33.3	538	29	3	0	67	0	1
	6	13.40	13.1	32	42.2	7.7	9.8	31.1	563	40	1	0	59	0	0
	14	13.73	13.1	31	42.2	8.9	9.5	31.0	301	33	1	0	64	0	2
Treated group															
	4	12.62	12.7	30	37.7	7.8	10.1	33.7	489	40	1	0	58	0	1
	1	12.23	12.3	31	37.5	9.0	10.1	32.9	550	41	2	0	57	0	0
	7	12.47	12.3	31	38.3	6.2	9.9	32.1	707	61	1	0	37	0	1
	12	10.98	10.8	31	34.4	10.1	9.8	31.4	558	38	4	0	58	0	0
	8	12.45	12.5	31	38.5	9.8	10.0	32.4	339	29	1	0	67	1	2
	5	13.54	13.1	32	43.8	8.1	9.7	29.8	345	49	1	0	50	0	0
	11	13.54	12.4	32	43.2	7.8	9.2	28.8	531	36	0	0	62	0	2

Legend: Hb – hemoglobin, MCV – mean corpscular volume, Ht – hematocrit, MCH – mean corposcular hemoglobin, MCHC – mean corposcular hemoglobin concentration. Haematological analyses were performed utilising the Scil Vet abc Plus (Horiba, Japan) automated haematological analyser. Differential white blood cell count was determined according to the standard procedure; smears were stained with Hemacolor (Merck, Darmstadt, Germany) and manually counted via microscopic examination. Blood samples for the haematological analyses were taken on 13. 10. 2017, haematological analyses were performed on 13. 10. 2017 and 18. 10. 2017.

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Table S4. Biochemistry results of the rams prior to the experiment.

	Ram Number	Urea (mmol/L)	Ca (mmol/L)	PO_4 (mmol/L)	Creatinine (μmol/L)	Cholesterol (mmol/L)	Triglycerides (μmol/L)	AST (μkat/L)	GGT (μkat/L)
Control group									
	10	4.5	2.6	2.69	64	1.8	0.4	1.83	0.88
	9	4.6	2.58	2.69	49	2.1	0.4	2.29	0.72
	13	5.2	2.79	2.48	80	2.1	0.3	2.41	0.93
	2	5.9	2.35	2.72	52	1.7	0.2	1.66	1.09
	3	5.1	2.58	3.05	54	1.5	0.5	2.28	0.98
	6	5.7	2.58	3.06	63	1.8	0.3	2.08	0.69
	14	6.2	2.62	2.83	56	1.7	0.2	2.95	1
Treated group									
	4	6	2.53	3.23	55	2	0.4	2.85	1.19
	1	4.8	2.48	3.93	62	2.1	0.3	2.6	0.92
	7	4.5	2.48	2.83	53	1.7	0.4	2.62	1.07
	12	4.2	2.4	3.25	53	1.8	0.5	2.08	1.14
	8	5.4	2.48	2.8	51	1.7	0.3	3.05	1
	5	5.9	2.56	2.53	69	2.7	0.3	2.54	0.97
	11	6.7	2.53	3.21	75	2.1	0.5	2.56	0.89

Legend: Ca – calcium, PO_4 – inorganic phosphate, AST – aspartate aminotransferase, GGT – gamma glutamyl transferase. For biochemistry analysis, automatic chemistry analyzer (Olympus Corp., Hamburg, Germany) was utilized.

S3. HPLC Analysis of Bisphenol A (BPA) in Blood Plasma Samples with Quality Assurance Procedures, Validation and Performance Characteristics

HPLC analysis

A 50 μL aliquot of the blood plasma samples was taken for the high-performance liquid chromatography (HPLC) analysis. A Hypersil GOLD C18 (150 × 4.6 mm, 3 μm particle size) analytical column was used which was protected by Hypersil GOLD 3μ drop in guard cartridges (Thermo Scientific, Waltham, MA, USA). The chromatographic process was performed at room temperature using a gradient HPLC method. The mobile phase was pumped at a flow rate of 1.0 mL/min and used the two constituents of a mobile phase, i.e., H_2O (constituent A) and MeCN (constituent B) in the following volume ratios: time 0–2 min (35% B), time 2–12 min (gradient 35–50% B), time 12–20 min (50% B), time 20–20.5 min (gradient 50–35% B) and time 20.5–21 min (35% B). The excitation and emission wavelengths of the fluorescence spectrophotometry analysis were set at 230 and 315 nm, respectively [61]. The results were evaluated according to an external standard method using a solvent standard calibration curve, which was constructed by plotting the peak area as a function of the analyte concentration.

Quality assurance procedures

Each sample series consisted of a baseline reagent sample, a baseline matrix sample, the study samples (in duplicate) and the two recovery samples. These were obtained by fortification of the baseline matrix sample with BPA on a reasonable level. Solvent standard calibration curves were constructed from 6 calibration points. The measured replicate study sample concentrations were corrected for the mean recovery rate of the respective

series, for an overall value of a baseline reagent sample, and the mean found concentration was used as a result.

Validation of BPA analysis

Validation was done separately for free and total BPA. Linearity was determined on a standard level by the least squares method, giving the regression and correlation parameters of the calibration lines. Solvent standard concentrations with 6 concentration points per calibration line ranged from 0.5–25 ng/mL in blood plasma. Recovery and intra-laboratory reproducibility of the method were tested by the response of the fortified matrix samples on separate time occasions. For free BPA determination in blood plasma these were fortified at 2 μg/L (n = 8) and for total BPA determination they were fortified at 10 and 20 μg/L (n = 8).

The precision of the methods was evaluated as the standard deviation and the coefficient of variation (CV) of the determined values and was assessed in accordance with the Horwitz coefficients (CV_H) according to Commission Decision 2002/657/EC [62]. The limit of quantification (LOQ) value was determined as the lowest analyte content for which the method proved acceptable in terms of recovery and repeatability.

Performance characteristics of BPA analysis

The analytical HPLC methodology used demonstrated good linearity, by the correlation coefficients' "R-squared" values of the solvent standard calibration lines of >0.998. The mean recovery values for determination of free BPA (fortification level of 2 μg/L) and total BPA (fortification levels of 10 and 20 μg/L) were 82.9% and 50.1%, respectively. The within-laboratory reproducibility of the measurements of the free and total BPA, represented by the CV values, were 10.5%, and 19.3%. The estimated LOQ values for determination of free and total BPA were 0.5 and 2 μg/L, respectively.

S4. Toxicokinetic Parameters of Rams Exposed to the First Dietary BPA Administration

Table S5. Toxicokinetic parameters of rams exposed to the first dietary BPA administration.

Ram No.	BW (kg)	C_{max} (μg/L)	T_{max} (h)	k_{el} (h-1)	$t_{1/2}$ (h)	AUC_t (μg · h/L)	AUC_i (μg · h/L)	AUMC (μg · h2/L)	MRT (h)	Cl (L/h/kg)	Vd (L/kg)
4	38.0	9.52	1.00	0.104	6.7	54.4	98.9	993.2	10.0	0.253	2.43
1	43.5	8.89	0.50	0.067	10.4	55.8	167.6	2802.6	16.7	0.149	2.24
7	44.5	10.69	1.00	0.078	8.9	58.5	119.3	1493.4	12.5	0.209	2.68
12	43.0	13.86	0.33	0.104	6.7	72.8	129.2	1279.0	9.9	0.194	1.86
8	47.0	9.15	1.00	0.086	8.1	51.1	99.9	1152.1	11.5	0.250	2.91
5	44.5	11.70	6.00	0.171	4.1	73.5	122.1	972.8	8.0	0.205	1.20
11	42.0	12.72	0.50	0.072	9.7	77.9	169.1	2296.3	13.6	0.148	2.06
n	7	7	7	7	7	7	7	7	7	7	7
Mean	43.2	10.93	1.48	0.097	7.8	63.4	129.4	1569.9	11.8	0.201	2.20
SD	2.8	1.90	2.01	0.036	2.2	10.9	28.8	707.3	2.9	0.042	0.57
CV (%)	6.4	17.4	136.5	36.6	27.9	17.2	22.3	45.1	24.4	21.1	25.9

n – number of rams, SD – standard deviation, CV – coefficient of variation, C_{max} – maximum plasma concentration, T_{max} – time of maximum plasma concentration, k_{el} – elimination rate constant, $t_{1/2}$ – elimination half-life, AUC – area under the curve, AUMC – area under the moment curve, MRT – mean residence time, Cl – clearance, Vd – volume of distribution.

S5. Results of Spermatozoa Analysis of Control and Treated Rams

Table S6. Results of spermatozoa analysis of the head of epididymis (mean ± standard deviation reported).

Examined Parameter	Control Group (n = 6)	Treated Group (n = 6)

CASA fresh		
ALH (μm)	2.108 ± 3.464	1.733 ± 3.668
ELO (%)	42.917 ± 13.922	51.583 ± 8.879
LIN (%)	43.167 ± 18.549	44.833 ± 13.615
Motility (%)	4.583 ± 5.229	1.083 ± 0.585
Progressive motility (%)	0.167 ± 0.408	0.000 ± 0.000
VAP (μm/sec)	38.292 ± 16.102	43.900 ± 9.672
VCL (μm/sec)	55.383 ± 27.356	62.208 ± 10.349
VSL (μm/sec)	29.025 ± 15.390	27.067 ± 10.947
HOST test (%)		
Dead	45.917 ± 6.320	44.000 ± 10.863
Live	54.083 ± 6.320	56.000 ± 10.863
Morphology of spermatozoa (%)		
Deformed acrosome	0.167 ± 0.408	0.000 ± 0.000
Detached acrosome	0 ± 0	0 ± 0
Acrosome in detachment	0 ± 0	0 ± 0
Distal droplet	11.000 ± 3.847	17.167 ± 7.885
Head abnormalities	0.5 ± 0.837	0.0 ± 0.000
Medial droplet	4.500 ± 3.564	5.333 ± 2.338
Mid-piece abnormalities	0 ± 0	0 ± 0
Multiple abnormalities	3.667 ± 3.938	3.500 ± 2.429
Neck abnormalities	0 ± 0	0 ± 0
Normal	13.000 ± 2.759	14.667 ± 7.633
Proximal droplet	66.333 ± 7.992	57.167 ± 14.959
Tail abnormalities	0.833 ± 0.408	2.167 ± 2.994
Spermatozoa concentration	122645833 ± 88744733	88541667 ± 26597159

Table S7. Results of spermatozoa analysis of the body of epididymis (mean ± standard deviation reported).

Examined Parameter	**Control Group (n = 6)**	**Treated Group (n = 6)**
CASA fresh		
ALH (μm)	7.050 ± 2.230	6.683 ± 1.481
ELO (%)	52.25 ± 3.205	48.25 ± 2.603
LIN (%)	39.250 ± 4.967	39.667 ± 4.143
Motility (%)	25.667 ± 15.075	24.083 ± 22.101
Progressive motility (%)	2.917 ± 3.513	3.750 ± 3.725
VAP (μm/sec)	61.183 ± 9.764	73.4167 ± 12.663
VCL (μm/sec)	102.383 ± 20.275	125.550 ± 30.341
VSL (μm/sec)	40.692 ± 8.789	51.717 ± 14.516
HOST test (%)		
Dead	36.167 ± 9.004	35.917 ± 10.938
Live	63.833 ± 9.004	64.083 ± 10.938
Morphology of spermatozoa (%)		
Deformed acrosome	0.167 ± 0.408	0.000 ± 0.000

Detached acrosome	0.333 ± 0.516	0.333 ± 0.516
Acrosome in detachment	0.500 ± 0.837	0.167 ± 0.408
Distal droplet	28.833 ± 17.314	36.000 ± 16.601
Head abnormalities	0.167 ± 0.408	0.333 ± 0.516
Medial droplet	1.833 ± 1.472	3.333 ± 2.066
Mid-piece abnormalities	0 ± 0	0 ± 0
Multiple abnormalities	27 ± 12.361	16 ± 4.195
Neck abnormalities	0.333 ± 0.816	0.000 ± 0.000
Normal	35.833 ± 11.392	34.167 ± 10.245
Proximal droplet	2.167 ± 1.329	1.667 ± 0.816
Tail abnormalities	2.833 ± 2.137	8.000 ± 8.414
Spermatozoa concentration	144843750 ± 156341692	112593750 ± 71684680

Table S8. Results of spermatozoa analysis of the tail of epididymis (mean ± standard deviation reported).

Examined Parameter	**Control Group (n = 6)**	**Treated Group (n = 6)**
CASA fresh		
ALH (μm)	7.633 ± 0.382	8.142 ± 0.816
ELO (%)	50.917 ± 2.223	49.833 ± 2.787
LIN (%)	41.417 ± 0.917	40.917 ± 5.643
Motility (%)	96.083 ± 1.068	84.667 ± 16.440
Progressive motility (%)	35.167 ± 5.125	30.417 ± 14.847
VAP (μm/sec)	97.383 ± 11.800	102.525 ± 11.580
VCL (μm/sec)	171.900 ± 21.052	180.225 ± 20.352
VSL (μm/sec)	72.342 ± 9.281	76.408 ± 11.812
HOST test (%)		
Dead	29.417 ± 7.664	33.000 ± 8.198
Live	70.583 ± 7.664	67.000 ± 8.198
Morphology of spermatozoa (%)		
Deformed acrosome	0 ± 0	0 ± 0
Detached acrosome	0.167 ± 0.408	0.167 ± 0.408
Acrosome in detachment	0.167 ± 0.408	0.333 ± 0.516
Distal droplet	34.5 ± 17.363	25.0 ± 11.628
Head abnormalities	0.333 ± 0.516	0.000 ± 0.000
Medial droplet	0.667 ± 0.816	0.500 ± 0.548
Mid-piece abnormalities	0 ± 0	0 ± 0
Multiple abnormalities	1.167 ± 1.941	1.500 ± 2.074
Neck abnormalities	0 ± 0	0 ± 0
Normal	59.5 ± 17.283	69.0 ± 14.339
Proximal droplet	2.167 ± 2.137	1.333 ± 1.751
Tail abnormalities	1.333 ± 1.506	1.667 ± 1.038
Spermatozoa concentration	644583333 ± 163473367	441458333 ± 37101353

Table S9. Results of spermatozoa analysis of the *ductus deferens* (mean ± standard deviation reported).

Examined Parameter	Control Group (n = 6)	Treated Group (n = 6)
CASA fresh		
ALH (μm)	7.65 ± 0.920	7.05 ± 0.694
ELO (%)	53.000 ± 5.030	53.333 ± 4.389
LIN (%)	41.250 ± 3.921	42.167 ± 4.844
Motility (%)	78.667 ± 27.531	55.333 ± 28.639
Progressive motility (%)	29.083 ± 11.320	22.000 ± 16.601
VAP (μm/sec)	95.15 ± 13.070	85.40 ± 16.087
VCL (μm/sec)	172.275 ± 26.578	151.991 ± 24.841
VSL (μm/sec)	73.233 ± 10.506	68.242 ± 15.490
HOST test (%)		
Dead	38.833 ± 7.488	44.500 ± 16.703
Live	61.167 ± 7.488	55.500 ± 16.703
Morphology of spermatozoa (%)		
Deformed acrosome	0.167 ± 0.408	0.167 ± 0.408
Detached acrosome	0.833 ± 0.408	0.0833 ± 0.204
Acrosome in detachment	0.233 ± 0.572	0.000 ± 0.000
Distal droplet	46.167 ± 6.646	27.583 ± 17.351
Head abnormalities	0.750 ± 0.987	0.167 ± 0.408
Medial droplet	1.000 ± 0.632	0.917 ± 1.114
Mid-piece abnormalities	0.083 ± 0.204	0.083 ± 0.204
Multiple abnormalities	2.667 ± 1.033	1.250 ± 1.172
Neck abnormalities	0.833 ± 1.602	0.667 ± 1.033
Normal	43.750 ± 7.069	63.833 ± 14.386
Proximal droplet	1.083 ± 1.497	0.583 ± 0.801
Tail abnormalities	2.667 ± 2.338	4.667 ± 3.266
Spermatozoa concentration	39694444 ± 38793064	27895833 ± 22256799

CASA: computer-assisted sperm analysis. ALH: amplitude of lateral displacement of the sperm head. ELO: elongation. LIN: linearity. VAP: average path velocity. VCL: curvilinear velocity. VSL: straight velocity. HOST: hypo-osmotic swelling test.