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Antenatale Glukokortikoid-Therapie, fetale Programmierung und die Rolle der Plazenta

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für das Fach Frauenheilkunde und Geburtshilfe

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1 Einleitung

Die Entwicklung des Kindes vor der Geburt beinhaltet eine ganze Reihe von komplexen, miteinander verknüpften Ereignissen, die sorgfältig aufeinander abgestimmt sein müssen, um eine optimale Gesundheit und Wohlbefinden für das ganze Leben zu ermöglichen. Die fetale Prägung beschreibt die Möglichkeit, wie in sensiblen Entwicklungszeitfenstern der Schwangerschaft durch bestimmte Faktoren Einfluss auf das Zellwachstum und die Entwicklung der Organe genommen wird, letztendlich mit dem Resultat einer anhaltenden postnatalen Veränderung in der Organ- und Gewebsfunktion.[1] Hierbei kann eine Fehlanpassung zu Erkrankungen im höheren Lebensalter führen („Fetale Programmierung“).

Bereits in den frühen 1970er Jahren wurden von dem Berliner Endokrinologen Günter Dörner an der Charité eine hormonabhängige prä- und neonatale Vorprogrammierung langfristiger Krankheitsrisiken und der darauf basierenden „Funktionellen Teratologie“ postuliert. Die Idee einer umweltabhängigen Ontogenese und Phylogenese weist natürlich eine längere Historie auf, und eine ausführliche Einordnung und Bewertung zur konzeptionellen und semantischen Historie wurde kürzlich von Andreas Plagemann hierzu verfasst.[2] In den frühen 1990er Jahren gelang es dem Epidemiologen David Barker und dem Biochemiker Nicholas Hales aufgrund von Beobachtungen über einen Zusammenhang von niedrigem Geburtsgewicht und dem vermehrten Auftreten von kardiovaskulären und metabolischen Erkrankungen im späteren Leben, einen Ansatz für die frühen Ursachen langfristiger Erkrankungen zu formulieren („Small-baby-Syndrom“).[3,4] Mit Entwicklung der „Match-Mismatch“ Hypothese durch Peter Gluckman und Mark Hanson[5] wurde das Prinzip der bereits in utero stattfindenden „prädiktiven Adaptation“ des sich entwickelnden Organismus auf unvorteilhafte Entwicklungsbedingungen als evolutionsbiologisch grundlegendes Prinzip vorgeschlagen.[5-8] Weltweit expandiert seither die Forschung auf dem Gebiet des „Developmental Origins of Health and Disease“ (DOHaD) rasant.

Ein übergreifendes Konzept der sog. „Vegetativen Prägung“ wurde von Andreas Plagemann bereits 2011 vorgestellt.[9] Hierunter versteht man, dass „...innerhalb eines vorgegebenen Entwicklungsfensters die vegetativen Grundfunktionen des werdenden Organismus auf die jeweils herrschenden Bedingungen (Hormone, humorale Faktoren u.a.) konditioniert werden. Diese werden als Norm völlig unbewusst, passiv und rezeptiv „verinnerlicht“. Mechanistisch geschieht dies grundsätzlich auf epigenomischer und mikrostruktureller Ebene. 'Vegetative Prägung' ist also ein Vorgang, der in der Entwicklung zu einem Abschluss kommt und beinhaltet das Postulat von Mechanismen…“ (zitiert aus A. Plagemann, *Naturwissenschaftliche Rundschau* 2014).[10] Im Gegensatz hierzu hebt der Begriff der „Perinatalen Programmierung“ auf die Entwicklungsphasen ab, „innerhalb der die Prägung stattfindet, und auf die Funktionsweise der durch Prägung erworbenen Mechanismen: (1) Die Perinatale Programmierung erfolgt in einer längeren Zeitspanne von der Konzeption bis etwa zum Abschluss des zweiten Lebensjahres“ … „(2) Sie bedeutet eine zur genetischen Disposition hinzukommende, individuell erworbene Entwicklungsvorgabe derart, dass der Organismus darauf programmiert

wird, fortan den durch die Vegetative Prägung verinnerlichten Normwert als Sollwert anzustreben. Epigenetische Muster wirken dabei fort und sind funktionell relevanter Bestandteil kybernetischer Regelkreise;…“ (zitiert aus A. Plagemann, *Naturwissenschaftliche Rundschau* 2014).[10]

Suboptimale intrauterine Bedingungen, wie zum Beispiel maternale Unterernährung, Hypoxie, psychischer Stress oder die *Glukokortikoid*-(GC) *Exposition* während der Schwangerschaft können die fetale Entwicklung nachhaltig beeinflussen und sind häufig mit einer Verringerung des Geburtsgewichts assoziiert. Die fetalen endokrinologischen Anpassungen während der Schwangerschaft an die veränderte intrauterine Umgebung haben in diesem Zusammenhang, im Gegensatz zur alleinigen Reduzierung des Geburtsgewichts, möglicherweise eine größere Bedeutung für das fetale Überleben und die langfristige Entwicklung. Der Zeitpunkt, an dem es zu Veränderungen der intrauterinen Umgebung kommt, scheint ebenfalls einen entscheidenden Einfluss auf die Art und das Ausmaß der gesundheitlichen Einschränkungen im Erwachsenenalter zu haben.[11-13] Das mögliche Spektrum der Spätfolgen ist breit und reicht von kardiovaskulären und stoffwechselbedingten Erkrankungen bis zur Ausbildung von malignen Tumoren im Erwachsenenalter.[14-21] Neugeborene mit niedrigem Geburtsgewicht haben so zum Beispiel ein erhöhtes Risiko, an einem Hepatoblastom (Lebertumor) im Alter zwischen 6 Monaten und 3 Jahren,[17] im fortgeschritteneren Lebensalter ein erhöhtes Risiko an einem Ovarial- oder einem Mammakarzinom zu erkranken.[19,20]

1.1 Fetale Programmierung und die Rolle von Glukokortikoiden

Eine wichtige Funktion bei der Organreifung und Differenzierung während der Schwangerschaft bei den meisten Säugetieren, hierin eingeschlossen der Mensch und das Schaf, nehmen GC mit Kortisol als ihrem Hauptvertreter ein.[22-24] Durch die physiologische Erhöhung der endogenen GC in einem bestimmten Entwicklungsfenster kommt es zur Reifung und Differenzierung zahlreicher Organsysteme, die für das spätere extrauterine Leben benötigt werden. Stressoren in der Schwangerschaft wie zum Beispiel psychischer Stress, Hypoxie, Frühgeburt oder maternale Unterernährung gehen mit Erhöhungen von endogenen GC einher und können somit zur fetalen Programmierung führen.[15,25] Es ist bekannt, dass maternale und fetale GC-Plasmaspiegel mit dem Geburtsgewicht korrelieren.[26-28] Der Schlüssel für das Verständnis der Rolle von GC für die fetale Programmierung liegt daher in der Beobachtung, dass im Tiermodell sowohl die fetale Wachstumsrestriktion als auch Komponenten des metabolischen Syndroms durch direkte hohe exogene GC-Exposition[29-31] oder durch vermehrte transplazentare Passage von endogenem Kortisol von der Mutter zum Feten (verminderte „11beta hydroxysteroid dehydrogenase typ 2“, 11ßHSD2-Aktivität)[32] induziert werden können. Ausser mit dem Geburtsgewicht korrelieren maternale und fetale GC-Plasmaspiegel ebenfalls mit dem neonatalen Blutdruck[33] und der Adipositas.[34]

Klinische Relevanz

Die Exposition des Feten gegenüber hohen exogenen GC-Spiegeln im Rahmen der Lungenreifeinduktion (LRI) bei drohender Frühgeburt hat ebenfalls direkte klinische Relevanz. Im Jahr 2010 wurden weltweit etwa 15 Millionen Kinder zu früh geboren, und rund eine Million dieser Kinder starben an den unmittelbaren Folgen der Frühgeburt.[35] Die Rate an Frühgeburt variiert hierbei und beträgt beispielsweise in Finnland 5%, Schweden 6%, Deutschland 9% und in den USA bis zu 12% aller Schwangerschaften und trägt etwa zu 75% der perinatalen Mortalität bei.[35,36] Die Frühgeborenen leiden häufig unter Krankheiten wie dem Atemnotsyndrom („respiratory distress syndrome", RDS), das sich aus der morphologischen und funktionellen Unreife der Lunge entwickeln kann. RDS allein ist für bis zu 60% der perinatalen Mortalität verantwortlich.[37]

Liggins & Howie[38] beschrieben als erste die Gabe von GC an Schwangere bei drohender Frühgeburt, um die Lungenreifung zu induzieren und die Mortalität und Morbidität aufgrund respiratorischer Störungen zu minimieren.[39] In zahlreichen Studien und Metaanalysen konnte die Wirksamkeit und die Verbesserung der neonatalen Morbidität und Senkung der Mortalität belegt werden. [37,38,40-42] Die Auswirkungen von GC auf die fetale Lunge sind vielfältig und betreffen neben den rein morphologischen Veränderungen mit anatomischer Lungenreifung auch die Expression von zahlreichen biochemischen Faktoren.[43] Funktionell wird durch die antenatale Gabe von GC die fetale Lungenreife induziert, die eine Verbesserung der Lungencompliance, eine Zunahme des maximalen Lungenvolumens, eine Abnahme der vaskulären Permeabilität, eine Reifung parenchymaler Strukturen, eine vermehrte Clearance von Lungenflüssigkeit vor der Geburt, eine Zunahme der Surfactantproduktion und ein besseres Ansprechen auf eine postnatale Surfactantbehandlung beinhaltet.[43]
Der Nutzen der antenatalen GC-Therapie zur LRI bei drohender Frühgeburt bei Einlingsschwangerschaften ist unbestritten, und neben der fetalen Lunge zeigen sich auch in anderen Organsystemen positive Effekte. Die Antenatale GC-Behandlung reduziert das Auftreten einer nekrotisierenden Enterokolitis („necrotising enterocolitis", NEC) signifikant um 54% (95% confidence interval (CI) 0.29 - 0.74), das Auftreten von Hirnblutungen („intraventricular hemorrhage", IVH) um 46% (95% CI 0.43 - 0.69), die Rate an neonatalen Infektionen innerhalb der ersten 48h um 44% (95% CI 0.38 - 0.85), das Risiko ein RDS zu entwickeln um 34% (95% CI 0.59 - 0.73), die neonatale Mortalität um 31% (95% CI 0.58 - 0.81) und die Notwendigkeit der stationären Aufnahme auf eine neonatologische Intensivstation („neonatal intensive care unit", NICU) um 20% (95% CI 0.65 - 0.99).[37] Hierbei ist die Anzahl der Frauen, die antenatal mit GC behandelt werden müssen, damit ein Kind von dieser Behandlung signifikant profitiert, unterschiedlich hoch (Number needed to treat (NNT), 95% CI): NEC 30 (23-29), Sepsis 27 (19-78), Perinatale Mortalität 23 (16-48), neonatale Mortalität 2 (16-36), IVH 21 (17-30), RDS 12 (10-15).[37,44]

Die „National Institutes of Health Consensus Development Conference" (1995/2001) kam zu dem Schluss, dass antenatale Gaben von GC zur fetalen Lungenreifeförderung mit einer Reduzierung der Mortalität, des

RDS und der Reduzierung von Hirnblutungen bei Frühgeborenen einhergehen, so dass die pränatale maternale GC-Gabe bei drohender Frühgeburt empfohlen wird.[45,46] Die Wahl des GC (Betamethason, BET; Dexamethason, DEX), die genaue Dosierung und das Intervall sind nach wie vor Gegenstand von Diskussionen.[47-58]

Die aktuelle Therapieempfehlung der Deutschen Gesellschaft für Gynäkologie und Geburtshilfe (DGGG; derzeit in Überarbeitung) empfiehlt die LRI bei drohender Frühgeburt mit BET in einem Gestationsalter zwischen 23+5 und 33+6 Schwangerschaftswochen (SSW). Hierfür soll die BET-Gabe von 2 x 12 mg intramuskulär im Abstand von 24 Stunden erfolgen.[60] Von einer routinemäßigen Wiederholung eines Zyklus antenataler GC bei Fortbestehen der Frühgeburt wird abgeraten.[60, 60] International werden jedoch unterschiedliche Empfehlungen zum Applikationszeitraum, der Wahl des GC sowie der Frage nach der Wiederholung der antenatalen GC-Therapie bei Fortbestehen der Frühgeburt >7 Tage nach GC-Erstgabe angeführt. So empfiehlt beispielsweise das Royal College of Obstetricians and Gynaecologists (RCOG) in seiner aktuellsten Leitlinie von 2010 den einmaligen Zyklus antenataler GC in einem Gestationsalter zwischen 24+0 und 34+6 SSW,[61] wobei auch eine GC-Gabe bei einer drohenden Frühgeburt zwischen 23+0 und 23+6 Wochen erwogen werden kann. Zusätzlich empfiehlt das RCOG die Gabe von antenatalen GC an alle Schwangere bis zur 38+6 SSW, bei denen die Indikation zum elektiven Kaiserschnitt gegeben ist.[61] Sollte der erste Zyklus vor der 26+0 SSW stattgefunden haben, wird eine Wiederholung empfohlen.[61] Entsprechend den letzten Empfehlungen des American Congress of Obstetricians and Gynecologists (ACOG) von 2011[62] könnte eine einmalige Zykluswiederholung der LRI in Betracht gezogen werden, wenn der erste Zyklus mehr als 2 Wochen zurück liegt, das Schwangerschaftsalter weniger als 32+6 SSW betrug und bei der Schwangeren weiterhin das Risiko besteht, innerhalb der kommenden 7 Tage zu gebären.

Ein weiteres Anwendungsgebiet der maternalen GC-Behandlung ist die Therapie des fetalen adrenogenitalen Syndroms in der Frühschwangerschaft, einer autosomal-rezessiven Störung der Kortisol-Synthese, die bei fehlender Substitution mit Kortisol zu einer Androgenisierung weiblicher Feten führt, sowie die chronische Gabe von GC bei maternalen Erkrankungen wie Asthma bronchiale oder Morbus Crohn.[63-66]

Betamethason vs. Dexamethason

Betamethason (BET) und Dexamethason (DEX) sind die einzigen beiden synthetisch hergestellten GC zur parenteralen maternalen Anwendung, die die Plazenta nahezu ungehindert, d. h. mit einer nur sehr geringen Affinität für 11ßHSD2, die maternales Kortisol in inaktives Kortison umwandelt, passieren können.[67] Andere GC wie zum Beispiel Hydrokortison oder Prednisolon erreichen zwar in entsprechend hohen maternalen Dosierungen ebenfalls die fetale Zirkulation, haben jedoch aufgrund der raschen Clearance nur eine sehr begrenzte Wirksamkeit.[68] Liggins und Howie verwendeten in Ihren ersten Versuchen eine Betamethason-Acetat-Suspension, die Aufgrund einer verlängerten Wirkungsweise etwa 30% der maternalen Wirkspiegel beim

Feten erreicht.[38] Wie bei BET[69] werden auch bei DEX nach maternaler Gabe im fetalen Serum etwa 30% der maternalen Serumspiegel erreicht.[70,71]

BET steht sowohl als Betamethason-Phosphat (BET-PO_4) als dephosphorylierte Form mit einer biologischen HWZ von 39-72h als auch als Betamethason-Acetat (BET-Ac) als deacitylierte Form mit einer HWZ 14h als Medikament von zur Verfügung.[52,72] Häufig werden Kombinationen aus beiden verwendet. Die längerfristige antenatale Gabe mit möglichst kontinuierlichem Erreichen von fetalen Wirkspiegeln scheint eine Voraussetzung für die Induktion der Lungenreife zu sein: So führte die einmalige fetale Injektion von sowohl BET-PO4, Dexamethason-Phosphat (DEX-PO4) sowie die maternale einmalige Injektion von BET-PO4 nicht zur einer LRI,[73-75] wohingegen wiederholte Gaben von Kortisol die Lungenreife induzieren.[75] Eine Kombination von BET-PO4 und BET-Ac scheint am wirkungsvollsten zu sein: Bereits eine einmalige antenatale maternale Gabe führt zu einer LRI beim Schaf, die sich durch eine zweite Gabe 24h nach der ersten Gabe signifikant steigern lässt.[73]

Im amerikanischen Sprachraum wurde DEX verwendet, da es für die internationalen Studien keine wirkliche gute Placebo-Alternative zu einer BET-Suspension gab.[52] DEX wird im Allgemeinen als DEX-Phosphat (DEX-PO4) mit einer biologischen Halbwertszeit von 36-72h angeboten.[43,52] Bezüglich ihrer Molekülstruktur unterscheiden sich beide lediglich in der Konfiguration einer Methylgruppe.[76] Allerdings verfügt BET im Gegensatz zu DEX über eine längere Halbwertszeit (HWZ), verminderte Clearance und ein größeres Verteilungsvolumen.[77] DEX erreicht niedrigere Spitzenpegel bei längerer biologischer Aktivität und schnellerer Blut-Hirnschrankenpassage.

Seit der NIH-Konsensus-Konferenz aus dem Jahr 1995 werden sowohl BET als auch DEX zur LRI empfohlen.[46] Obwohl es bislang keine sicheren Beweise oder einen akzeptierten Konsens darüber gibt, BET gegenüber DEX zur antenatalen LRI zu empfehlen,[78] haben kürzlich veröffentlichte Studien ein geringeres Risiko für eine zystische periventrikuläre Leukomalazie (PVL) bei Frühgeborenen nach BET-Exposition gezeigt (OR 0,3; 95%CI 0,1-0,7).[79,80] Diese Reduktion konnte nicht nach einer DEX-Behandlung festgestellt werden, insbesondere wenn mehrere Zyklen gegeben wurden.[79] Andere Studien fanden in der BET-Gruppe im Vergleich zu DEX eine geringere neonatale Mortalität (OR 0,44 BET vs. 0,73 DEX),[81] sowie eine größere Reduktion der RDS-Häufigkeit (BET: OR 0,56 95% CI 0,48-0,65 vs. DEX OR 0,80 95% CI 0,68-0,93).[37] Eine Studie von Egermann et al. dokumentierte sogar nach DEX-Therapie eine erhöhte Rate an neonataler Sepsis (RR 8.48, 95% CI 1,11–64,93).[82] In einer umfangreichen Cochrane-Analyse von Brownfoot et al. 2013, in der 12 Studien mit 1557 Frauen eingeschlossen werden konnten, wurden BET und DEX sowie verschiedene Applikationsformen und Darreichungswege im Hinblick auf das neonatale Outcome untersucht.[47] Nach DEX im Vergleich BET gab es weniger IVH (Risikoverhältnis (RR) 0,44, 95% Cl 0,21-0,92).[47] Keine statistisch signifikanten Unterschiede bestanden bzgl. der anderen primären Endpunkte wie dem RDS (RR 1,06 95% Cl 0,88-1,27) oder dem perinatalen Tod (RR 1,41, 95% CI 0,54-3,67).[47] Sekundäre Endpunkte wie dem Intervall Geburt - Verlegung auf die

NICU oder bestimmte biophysikalische Parameter zeigten keine klinisch bedeutsamen oder signifikante Unterschiede.[47] Neben einer reduzierten mütterlichen postpartalen Aufenthaltsdauer für Frauen, die BET im 12-Stundentakt im Vergleich zum 24-Stundentakt in einer Studie erhalten hatten (MD -0,73 Tage, 95% CI -1,28 bis -0,18), fanden sich keine weiteren signifikanten Unterschiede in Bezug auf die verschiedenen BET-Dosierungsintervalle.[47] Ebenso wurden keine signifikanten Unterschiede im Vergleich von BET-Ac plus BET-PO4 vs. nur BET-PO4 gefunden.[47] Die Autoren heben hervor, dass es weiterhin unklar verbleibt, welches GC bzw. welches Dosierungsregime zu bevorzugen sei. Weitere Studien laufen derzeit, um die Rolle von maternalem DEX gegenüber BET zur Prophylaxe der mit der Frühgeburt-assoziierten Morbidität und im Hinblick auf das Überleben von Kindern ohne Behinderung zu evaluieren.[83]

Dosierung

Die optimale Dosierung der antenatalen GC-Therapie, der optimale Zeitpunkt und die Frage der Wiederholung und der damit einhergehenden Dosiserhöhung bleiben weiterhin ungeklärt. Derzeit werden für BET 2x12 mg intramuskulär im Abstand von 24h und für DEX 4x6 mg intramuskulär im Abstand von 48h empfohlen.[84] Insbesondere die Wiederholung und damit die Erhöhung der Gesamtdosis bei Fortbestehen der drohenden Frühgeburt[85,86] oder bei Zwillingen[87] ist weiterhin Gegenstand intensiver Diskussionen.[47,50,51,53]

Bis Ende der 1990er Jahre war die repetitive Gabe von antenatalen GC zur LRI mit Wiederholungen alle 7 Tage nach dem ersten BET-Zyklus gängige Praxis.[88,89] Hintergrund hierfür waren tierexperimentelle Studien am Schaf, die eine maximale fetale Surfactantproduktion erst nach über Wochen wiederholten GC-Gaben zeigten[90] sowie in-vitro-Untersuchungen an Zelllinien von Pneumozyten, in welchen die durch DEX-stimulierte Surfactantprotein-B-mRNA-Expression am Tag 8 nach Gabe wieder auf die Werte von nicht behandelten Kontrollen zurückging.[91] Des Weiteren wurde in einer Subgruppenanalyse in der ersten Studie zur antenatalen LRI mit GC erkannt, dass die BET-Gabe bei Feten, die länger als 7 Tage nach der ersten BET-Gabe geboren wurden, keine verbesserte Lungenfunktion mehr hatten.[92,93] Morphologische Untersuchungen zeigten jedoch, dass die biochemischen und auch strukturellen Reifungsveränderungen der fetalen Lungen für mindestens 2-3 Wochen nach der BET-Gabe nachweisbar waren und erst dann eine langsame graduelle Rückbildung stattfand.[39,94-96] In der später durchgeführten Untersuchung von Howie und Kollegen konnte bei einer Erhöhung der BET-Gesamtdosis (2x24mg) keine zusätzliche Verbesserung der neonatologischen Entwicklung beobachtet werden.[92] In einer großen Cochrane-Datenanalyse an 10 Studien und insgesamt 5700 Neugeborenen konnte gezeigt werden, dass die wiederholten Gaben von GC zur LRI mit einer signifikanten Reduzierung der Inzidenz von RDS und schwerer neonatalogischer Morbidität im Vergleich zu der Einmalgabe assoziiert waren.[48] Der absolute Nutzen von wiederholten Gaben war dem der Einzelgabe gleich zu setzen („number to treat to prevent RDS“, NNT (95% CI): Einzelgabe GC 12 (10-19)[37] vs. repetitiven Gaben 17 (11-32)).[86] Der Zeitpunkt der LRI scheint einen Einfluss zu haben: Feten profitieren von wiederholten GC-Gaben besonders, wenn diese vor 27+6 Woche gegeben werden.[97]

Insbesondere hohe exogene GC-Dosen im Rahmen der früher üblichen repetitiven Gabe von antenatalen GC zur LRI kann erheblichen Langzeitfolgen haben (Tabelle 1), wobei sich Unterschiede bzgl. des Geschlechts, der Tierspezies, dem Stadium der Organentwicklung und der Applikationsdauer zeigen.

Tier:	Referenzen:	Mensch:	Referenzen:
⇓ Fetalwachstum (Geburtsgewicht, Kopfumfang, Körperlänge etc.)	330,39,98-109	⇓ Fetalwachstum (Geburtsgewicht, Kopfumfang, Körperlänge etc.)	37,40,48,105,110-123
⇓ Plazentagewicht	98,108,124-135	⇓ Plazentabreite	123
⇑ Beeinträchtigung der HPA-Achsen Funktion	102,104,136-154	⇑ Beeinträchtigung der HPA-Achsen Funktion	69,146,155-173
⇓ Fortbewegung, Leistungswille, Wahrnehmungsfähigkeit	140,174	⇑ Neuropsychiatrische und Verhaltensveränderungen	158,175-178
⇓ Nerven-Myelinisierung	141,145,148,150,179,180		
⇑ Beeinträchtigung des Stoffwechsels (Adipositas, Hyperinsulinämie, Hyperglykämie, Insulinresistenz etc.)	30,99,139,143,151,165,181-184	⇑ Beeinträchtigung des Stoffwechsels (Adipositas, Hyperinsulinämie, Hyperglykämie etc.)	117,185-187
⇑ Herz-Kreislauf-System (Hypertension)	198-21429,104,139,183,188-195	⇑ Herz-Kreislauf-System (Hypertension)	158,186,187,189,196-200
⇓ Fruchtbarkeit	142	⇑ Fetale Herzfrequenzvariation	201,202
⇓ Nephrone	190,194,203-206	⇓ Nierenfunktion (GFR)	187

Tabelle 1: "Assoziierte Effekte der antenatalen GC-Therapie im Tiermodell und beim Menschen". Zusammengetragen aus Braun et al. Endocrine Reviews 2013[15] und McKinlay et al. 2014[44]

Letztendlich ist bislang aber noch keine abschließende Bewertung zu der Dosierung und der Frage der Wiederholung erfolgt.[46] Sowohl wir[116] als auch andere[52,207] stellen jedoch die derzeit wohl überwiegend praktizierte Dosierung in Frage und vermuten, dass die aktuelle Empfehlung mit 2x12mg BET zu hoch ist. Im Tiermodell konnte zum Beispiel gezeigt werden, dass bereits die einmalige Gabe von 12mg BET ausreicht, um die Lungenreife zu induzieren und sowohl die kardiovaskuläre als auch renale Funktion beim Schaf zu verbessern.[208,209] Bereits 15h nach der Gabe von BET ist mit einer Verbesserung der Lungenfunktion zu rechnen.[210]

Wirkungsweise von GC am Glukokortikoidrezeptor

Das Verständnis der Einflussnahme von endogene GC (Kortisol) als auch die exogenen GC (BET, DEX) auf die Organreifung beginnt mit der Funktion des Glukokortikoidrezeptors (GR). Dieser gehört zu der Familie der Kernrezeptoren, die intrazellulär am Zellkern lokalisiert sind und als ligandenabhängige Transkriptionsfaktoren[211] somit die Transkription[212] als auch die mRNA-Stabilität[213] und posttranslationalen Prozesse[214] beeinflussen. Nachfolgende Signalwege können entweder direkt durch den aktivierten GR via Bindung an Kernrezeptorelemente („nuclear response elements", NRE) innerhalb spezifischer Gen-Promotorregionen[213,215-219] oder durch Interaktion mit Transkriptionsfaktoren[212] aktiviert werden. Die ligandenabhängige GR-alpha-(GR_α) Isoform stimuliert im Zielgewebe die Transkription und wird als aktive Form beschrieben.[220] Mehr als

acht verschiedene GRα-Isoformen wurden bislang beschrieben, darunter GRα-A (94kDa), GRα-B (91kDa), GRα-C1-C3 (81-83kDa) und GRα-D1-D3 (50-55kDa).[221] Alle GRα-Isoformen regulieren als Kernrezeptoren die zelluläre Transkription. Hinter den verschiedenen Splice-Varianten vermutet man eine unterschiedliche biologische Aktivität bzw. Reagibilität auf verschiedene physiologische Stimuli.[222] Bei der ligandenunabhängigen GR_{β} Isoform vermutet man einen hemmenden Wirkungsmechanismus auf den GRα.[223] Die Splice Varianten GRɣ (95kDa), GR-A (65kDa) und GR-P (75kDa) besitzen nur ein geringgradiges Transkriptionspotenzial.[224,225] Neben den direkten genomischen Wirkungen verfügen GC insbesondere in höheren Dosierungen auch über nicht-genomische Effekte, wie zum Beispiel eine Beeinflussung der Zellpermeabilität, der Mitochondrienfunktion oder von intrazellulären Signalwegen.[226-228]

1.2 Fetale Programmierung der HPA-Achse

Während der Fetalentwicklung wird Kortisol in den fetalen Nebennieren als Antwort der Hypothalamus-Hypophysen-Nebennieren Achse („hypothalamus pituitary adrenal axis", HPA-Achse) gebildet. GC, mit Kortisol als seinem Hauptvertreter, besitzen eine wichtige Funktion in der Regulation der Organentwicklung und der Organreifung bei den meisten Säugetieren, hierin eingeschlossen der Mensch und das Schaf.[24]

Die HPA-Achse gliedert sich in den Hippocampus, den Hypothalamus, die Hypophyse und die Nebennierenrinde. Das in den peripheren Blutkreislauf abgegebene Adrenocorticotropin (ACTH) führt in der Nebennierenrinde zur Freisetzung von GC.[229,230] Der hauptsächliche Stimulus für die Ausschüttung von Kortisol ist ACTH. Allerdings modulieren auch verschiedene Hormone des Nebennierenmarks wie zum Beispiel Noradrenalin die GC-Sekretion.[231]
Während der Schwangerschaft werden die fetalen Kortisol-Spiegel in einem engen Bereich reguliert, um normales Wachstum zu ermöglichen. Kurz vor der Geburt kommt es beim Feten zu einer Aktivierung der HPA-Achse mit einer vermehrten Freisetzung von Kortisol (bei Nagetieren Kortikosterone) in die fetale Zirkulation.[232] Durch die physiologische Erhöhung der GC in einem bestimmten Entwicklungsfenster kommt es zur Reifung und Differenzierung der Organsysteme, die für das spätere extrauterine Leben benötigt werden.[232-234] Der deutliche Anstieg von fetalem Plasmakortisol gegen Ende der Schwangerschaft ist hierbei speziesübergreifend zu beobachten und mit einem Anstieg der fetalen Plasmakonzentration von ACTH assoziiert.[184,235-238]

Das Verständnis der molekularen Mechanismen der fetalen HPA-Achsenaktivierung in Terminnähe beruht vornehmlich auf Untersuchungen im Schaf.[162,164,236,239] Gegen Ende der Schwangerschaft zeigt sich im Nucleus paraventricularius (PVN) ein Anstieg der mRNA-Expression von Arginin-8-Vasopressin (AVP) und Corticotropin freisetzendes Hormon (CRH), der zu einem temporären Anstieg von Proopiomelanocortin (POMC) im fetalen Anteil des Hypophysevorderlappens führt.[240,241] Die hieraus resultierende Ausschüttung von ACTH führt

sowohl zu einer vermehrten Expression von ACTH-Rezeptoren (MC2-R) in der fetalen Nebennierenrinde als auch zu einer vermehrten Aktivierung der Schlüsselenzyme in der adrenalen Kortisol-Synthese.[240,241] Der Fetus selbst reagiert auf ein verändertes intrauterines Milieu mit einer vorzeitigen HPA-Achsenaktivierung mit frühzeitiger Aktivierung von Schlüsselenzymen entlang der gesamten HPA-Achse. Daher können Veränderungen in utero, die mit erhöhten endogenen Kortisol-Spiegeln einhergehen, in sensiblen Entwicklungsphasen zu einer langfristig veränderten Organentwicklung und Organreifung führen.

GC-Exposition und die fetale HPA-Achse

Sowohl im Tier als auch beim Menschen konnte wiederholt gezeigt werden, dass Veränderungen im intrauterinen Milieu auf die exakt abgestimmten Entwicklungs- und Reifungsprozesse der HPA-Achsenentwicklung durch Modifikation der Schwelle der negativen Rückkopplung Einfluss nehmen. Diese Modulation wird häufig durch Änderungen in zentralen Rezeptorpopulationen, Enzymaktivität und Expression verursacht.[102,242-246] Stressoren in der Schwangerschaft, wie zum Beispiel psychisch-sozialer Stress, Hypoxie, Frühgeburt oder maternale Unterernährung gehen mit Erhöhungen von endogenen GC einher.[25] Diese Stressoren setzen den Feten überhöhten endogenen GC-Spiegeln aus. Die pränatale Gabe von synthetischen GC ist ein etabliertes Modell zur Untersuchung der Auswirkungen von „Stress“ in der Schwangerschaft und dessen GC-vermittelten Prägungseffekten.[247-253]

In einer Vielzahl von Tierspezies (Schaf, Ratte, Meerschweinchen und Primat) konnten nach antenataler GC-Gabe Veränderungen in der fetalen HPA-Achsenfunktion nachgewiesen werden.[161,192,193,195-200] Die Ausprägung variiert dabei in Abhängigkeit vom gewählten Präparat (BET, DEX), dem Applikationszeitpunkt in der Schwangerschaft, der Gesamtdosis sowie dem Geschlecht.[136,142,145-148] Beim Schaf zum Beispiel führte die einmalige antenatale BET-Exposition im letzten Schwangerschaftsdrittel zu einer Erhöhung der fetalen ACTH-Basalspiegel sowie der Kortisol-Bindungskapazität ohne eine Beeinträchtigung der hypothalamischen bzw. hypophysären POMC- oder CRH-mRNA-Expression.[102] Im Alter von einem Jahr zeigten sich bei den Schafen in demselben Modell sowohl signifikant höhere Kortisol-Basalspiegel als auch ein vermehrtes Ansprechen auf einen ACTH-Stimulus.[137] Höhere BET-Dosierungen hingegen verminderten das ACTH-Ansprechen im Alter von 6 Monaten und 1 Jahr.[137]

Es gibt nur wenige Studien zu den Auswirkungen der antenatalen GC-Behandlung auf langfristige Veränderungen der HPA-Achse beim Menschen.[160] Die antenatale GC-Behandlung führte so zu einer reduzierten HPA-Achsenaktivität beim Neugeborenen, wobei die GC-Basalspiegel im Erwachsenenalter keine signifikanten Veränderungen aufwiesen.[146] Insbesondere der Zeitpunkt der antenatalen GC-Exposition in Bezug auf den Entwicklungsstand der fetalen HPA-Achse scheint hierbei eine Bedeutung zu bekommen. Untermauert wird diese Annahme durch eine Studie an einjährigen Kindern, die nur dann niedrigere Speichelkortisol-Spiegel

aufwiesen, wenn die pränatale Stressexposition (bestimmt am Beispiel der 9-11-World-Trade-Center-Katastrophe) im letzten Schwangerschaftsdrittel auftrat.[254] Andere Arbeitsgruppen konnten zeigen, dass lediglich in der ersten Woche nach antenataler GC-Exposition die basalen Kortisol-Plasmaspiegel erhöht waren, diese sich anschließend aber wieder normalisierten.[69,167-170]

Die Langzeitfolgen der fetalen Programmierung der HPA-Achse sind bislang noch unklar. In einer Untersuchung an dreißigjährigen nach antenataler GC-Exposition wurden 7% höhere morgendliche Kortisol-Plasmaspiegel im Vergleich zu den Kontrollen gemessen, wobei dieser Unterschied nach Adjustierung für Konfounder nicht mehr signifikant war.[117] Dennoch lässt diese Beobachtung die Vermutung aufkommen, dass die antenatale GC-Behandlung nicht nur die fetale HPA-Achsenfunktion beeinflusst, sondern eben auch mit langfristigen endokrinologische Veränderungen einhergeht.[146,160] Häufig gehen die Veränderungen nach GC-Exposition mit veränderter Entwicklung der fetalen HPA-Achse mit einer Wachstumsrestriktion einher, das heißt, der Fet hat sein genetisch determiniertes Wachstumspotenzial nicht realisiert („intrauterine growth restriction“, IUGR).[137,242,255,256] IUGR selbst ist mit erhöhtem fetalen[257] ACTH und Kortisol und erhöhten Kortisol-Spiegeln im Erwachsenenalter assoziiert.[258-260] Die mit einer veränderten intrauterinen Umgebung einhergehende fetale Prägung der HPA-Achse wird als entscheidend für die Entstehung von Erkrankungen im Erwachsenenalter wie zum Beispiel der Herzinsuffizienz,[16] Insulinresistenz,[261,262] Typ-2-Diabetes mellitus[263,264] und Hypertonie[265,266] angesehen.

1.3 Fetale Programmierung und die Rolle der Plazenta

Die Plazenta übernimmt während der Schwangerschaft eine einzigartige Rolle in der Unterstützung des Feten. Sie schützt den Feten vor einer immunologischen Abwehrreaktion des mütterlichen Organismus und sorgt gleichzeitig für den Nährstoffaustausch zwischen Mutter und Fetus. Zusätzlich produziert die Plazenta Peptid- und Steroidhormonen, die den plazentaren, maternalen und fetalen Stoffwechsel und ihre Entwicklung beeinflussen. Bei Säugetieren stellt die Nährstoffversorgung durch die Plazenta für den Feten den limitierenden Faktor für das intrauterine Wachstum dar.[267] Sie ist abhängig von der Plazentagröße, der Morphologie, der Blutversorgung, dem Vorhandensein von Transportermolekülen und der Fähigkeit der Plazenta, Nährstoffe selbst zu verstoffwechseln und Hormone zu produzieren. Eine Beeinflussung der Fetalentwicklung bzw. eine Adaptation des Feten an eine veränderte intrauterine Nährstoffversorgung durch die Plazenta kann mit kardiovaskulären Erkrankungen und Stoffwechselstörungen im Erwachsenenalter einhergehen.[268] Epidemiologische Studien an Mensch und Tier weisen darauf hin, dass maternale Unterernährung die Nachkommen für Erkrankungen im Erwachsenenalter prägt. Dieser Effekt könnte direkt, oder aber indirekt, z. B. durch Funktions- und Strukturveränderungen in der Plazenta bedingt sein.[269]

1.4 Interaktion - Plazenta und fetale HPA-Achse

In den meisten Modellen der „early life adversity“ führen mütterlicher Stress, exogene GC-Gaben, experimentell induzierte plazentare Insuffizienz sowie Hypoxie zu maternal erhöhten GC-Spiegeln.[22,270,271] Aufgrund der terminnahen Aktivierung der fetalen HPA-Achse kommt es zum Anstieg der fetalen Kortisol-Spiegel.[23,292] Somit ist die Plazenta sowohl den GC-Signalen der Mutter als auch denen des Feten ausgesetzt.[233,272-275] Etwa 80% der fetalen GC stammen, noch bevor es zur Aktivierung der fetalen HPA-Achse kommt, von der Mutter,[276] wobei die fetalen Kortisol-Konzentrationen etwa 2/3 niedriger sind als die maternalen. Der transplazentaren Kortisol-Transfer von der Mutter zum Feten wird hierbei durch das Kortisol metabolisierende Enzyms 11ßHSD2, das das aktive maternale Kortisol in das inaktive Kortison umwandelt, reguliert.[271,277-281] Neben der Expression von 11ßHSD2 in der Plazenta findet sich 11ßHSD2 auch in zahlreichen anderen fetalen Organen wie zum Beispiel der Leber, der Niere und den Lungen und reguliert somit auch in anderen Organen die lokalen Kortisol-Spiegel.[282-284]

Im Vergleich zur Ratten- und Mausplazenta, wo man einen Abfall der 11ßHSD2-Aktivität gegen Ende der Schwangerschaft findet und somit zu einem vermehrten transplazentaren Kortisol-Transfer von der Mutter auf den Feten und zu einer Organ- und Lungenreifung führt,[337,338] bleibt beim Menschen die 11ßHSD-Aktivität nahezu gleich.[27] Die endogene Kortisol-Exposition des Feten während der Fetalentwicklung wird durch das plazentare 11ßHSD2 in utero reguliert, folglich scheint es eine wichtige Rolle bei der GC-induzierten fetalen Programmierung einzunehmen. Pränatale Einflüsse, die die Aktivität von 11ßHSD2 in der Plazenta beeinflussen, könnten so den Feten im Falle einer 11ßHSD2-Aktivitätsminderung einem erhöhtem endogenen Kortisol-Transfer aussetzen, der zu einer Beeinträchtigung des fetalen Wachstums führt.[26,27,285]

Die Aktivität von 11ßHSD2 wird durch eine Vielzahl von Faktoren beeinflusst; zum Beispiel zeigt sich eine Verminderung der plazentaren 11ßHSD2-Aktivität nach maternalem Stress, bei maternaler proteinarmer Ernährung, erniedrigten maternalen Sauerstoff-Leveln, erhöhten Katecholaminen oder Exposition gegenüber inflammatorischen Zytokinen.[286-291] Im Rattenmodell besteht eine Zusammenhang von niedrigen 11ßHSD2-Spiegeln mit Hypertension im Erwachsenenalter.[279] Ebenfalls im Rattenmodell führte eine Behandlung mit einem 11ßHSD2-Inhibitor (Carbenoxolon) zu einer signifikanten Reduktion des Geburtsgewichts, erhöhten Nüchternblutzuckerwerten, einer vermehrten Reagibilität der Insulinausschüttung nach Glukosebelastung und erhöhten Kortikosteron-Werten, assoziiert mit gesteigertem Fluchtverhalten.[207,346,347] Diese Veränderungen konnten durch eine maternal Adrenalektomie aufgehoben werden, was die Rolle der mütterlichen Kortisol-Spiegel für die Induktion fetaler Programmierung untermauert.[151]

Auch beim Menschen konnte ein Zusammenhang zwischen plazentaren 11ßHSD2-Spiegeln und dem fetalen Wachstum gefunden werden. Eine verminderte plazentare 11ßHSD2-Aktivität führt zu einer fetalen Wachstumverminderung.[26,348] Feten von Schwangeren mit einer homozygoten Mutation des 11ßHSD2-Gens weisen

ein vermindertes Geburtsgewicht auf. [292] Übermäßiger Konsum von Lakritz (Inhaltsstoff: Glycyrrhetinsäure) in der Schwangerschaft führt zu einer Inhibierung der plazentaren 11ßHSD2-Aktivität, die mit einer verkürzten Schwangerschaftsdauer, signifikant verminderten verbalen Fähigkeiten sowie Verhaltensauffälligkeiten und Aggression assoziiert war.[197,293-296] Interessanterweise zeigte sich auch eine niedrigere 11ßHSD2-Aktivität im Trophoblasten von weiblichen im Vergleich zu männlichen Plazenten.[65] Möglicherweise führen bei männlichen Feten erst wesentlich höhere maternale Kortisol-Spiegel zu einer Beeinträchtigung der fetalen HPA-Achsenentwicklung, und eine autonome Entwicklung im Vergleich zu den weiblichen Feten wäre über wesentlich höhere endogen anflutende maternale Kortisol-Spiegel möglich.[64] Spekulativ, aber wahrscheinlich ist auch, dass sich die geschlechtsspezifischen Veränderungen, die im Rahmen der fetalen Programmierung beobachten werden, auf geschlechtsspezifische Unterschiede in der plazentaren 11ßHSD2-Aktivität zurückführen lassen.

Es wird postuliert, dass die maternale HPA-Achse die Regulation der bioaktiven GC-Spiegel und die Veränderungen in der fetalen HPA-Achsen Funktion miteinander interagieren. Ein Mechanismus könnte die Fähigkeit der Plazenta sein, Neuropeptide zu sezernieren. Eines dieser Neuropeptide ist das plazentare CRH.[297] Plazentares CRH steigt mit dem Schwangerschaftsalter an und ist besonders im Rahmen einer Frühgeburt deutlich erhöht. [298] Die Genexpression und Peptidsekretion ist hierbei Kortisol abhängig.[297] Zytokine, Stickstoffmonoxid, Oxytocin und Progesteron können ebenfalls die plazentare CRH-Sekretion modulieren.[297] Daher könnte es im Fall einer frühen „early life adversity“ mit vermehrter Ausschüttung von maternalem Kortisol zu einer vermehrten Freisetzung von plazentarem CRH kommen.

Ähnlich dem CRH lässt sich ebenfalls Urokortin als ein weiteres Mitglied der Kortikotropin Familie in der Plazenta nachweisen.[299] Ein vermindertes Sauerstoffangebot führt in der Zellkultur zu einer verminderten Urokortin-2 und -3 Expression.[300] Urokortin selbst stimuliert die plazentaren Aromatasen und induziert die Bildung von plazentarem Estradiol[301] und hat somit eine Möglichkeit, im Sinne eines alternativen Regulationsweges den plazentaren Blutfluss reaktiv auf eine verändertes intrauterines Milieu anzupassen.[302] Auch interagiert plazentares Urokortin mit Lipopolysachariden (LPS), die die Freisetzung von pro-inflammatorischen Zytokinen in vitro und in vivo induzieren.[303] Dazu passt die Beobachtung, dass die Plazenta von Frauen mit drohender Frühgeburt und Chorionamnionitis signifikant höhere Spiegel an CRH, Urokortin-2 sowie CRH-1 Rezeptoren aufweisen.[303] Man vermutet, dass geringgradige plazentare Infektionen das fetale Wachstum über diesen Regulationsweg hemmen und somit zur Frühgeburt prädisponieren; ein veränderter Signalweg in der Immunantwort könnte einen gemeinsamen Mechanismus beider Pathologien darstellen.[355,361]

1.5 Tiermodell Schaf

Das Schaf und andere Tiermodelle wie Ratte, Maus, Meerschweinchen und Primaten werden zur Untersuchung der verschiedenen Aspekte der fetalen Programmierung genutzt. Jedes Tiermodell stellt ein mehr oder weniger gutes Abbild für die Entwicklung beim Menschen dar.[304-306] Trotz gewisser Einschränkungen in der direkten Übertragung der Ergebnisse auf den Menschen ist das Schaf für die Untersuchung von maternalem Stress und der antenatalen Exposition mit GC auf die Fetalentwicklung ein sehr gut untersuchtes und etabliertes Tiermodell.[304,305,307-311] Im Gegensatz zu anderen Tiermodellen (Kaninchen, Ratte, Maus) findet die Ausbildung der Alveolen in der Lunge beim Schaf etwa im gleichen Schwangerschaftsalter wie beim Menschen statt (Mensch 80% vs. Schaf 75% des Schwangerschaftsalters), und auch die Körpergewichtszunahme zu diesem Zeitpunkt ist ähnlich (Mensch: 1,6% vs. Schaf 3,8%).[312] Mit diesem Wissen lassen sich Unterschiede zwischen den einzelnen Modellen in Bezug auf die Ausprägung gewisser Veränderungen nach antenataler GC-Gabe besser erklären. So zum Beispiel führt eine antenatale LRI beim Schaf im Alter von 105 Tagen (=70% Schwangerschaftsdauer) zu einer Geburtsgewichtsreduktion von 11%, was einem Wachstumsarrest von etwa vier Tagen entspricht.[313] Würde man diese Daten auf den Menschen übertragen, würde sich bei einem ähnlichen Applikationszeitpunkt (~28. SSW) nach antenataler LRI mit GC eine Geburtsgewichtsreduktion von etwa 4,8% zeigen, die allerdings sicherlich nur schwer zu detektieren wäre.[312] Beachtenswert ist weiterhin, dass zum Beispiel ein einzelner Kurs mit GC über 48h im Mausmodell aufgrund der kurzen Schwangerschaftsdauer von 18 Tagen bereits einen Anteil von 11% Schwangerschaftsdauer entspricht, hingegen beim Mensch lediglich 0,8% und beim Schaf 1,3%. Daher eignen sich Tiermodelle mit einer kurzen Schwangerschaftsdauer weniger, um die Effekte antenataler GC auf die Fetalentwicklung zu untersuchen.

Studien zur Gefäßversorgung in der Plazenta bei Mensch und Schaf haben gezeigt, dass trotz Unterschiede in der Morphologie und Ultrastruktur der Plazenta die Schafsplazenta für die menschliche Plazenta ein geeignetes Tiermodell darstellt.[306,314] Im Gegensatz zur menschlichen, hämochorialen Plazenta besitzt das Schaf eine synepitheliochoriale Plazenta mit einer nicht-invasiven Plazentation.[315] Der zweihörnige Uterus des Schafs besitzt etwa 60-80 Karunkel, die zur Anlage der maternofetalen Einheiten, den sogenannten Plazentome, zur Verfügung stehen.[316] Historisch wurden die Plazentome gemäß ihrem morphologischem Erscheinungsbild, dem Grad des Ektropiums der sich dunkel darstellenden „Hämophagus-Zone“, in vier Typen A-D unterteilt.[316] Älteren Studien behaupten, es gäbe im Verlauf der Schwangerschaft eine kontinuierliche Umwandlung von A- über B, C in D-Plazentome.[317-321]

Bei der Interpretation und der Übertragung von Ergebnissen aus dem Tiermodell auf den Menschen ist zu berücksichtigen, dass dabei in einer gut kontrollierten Umgebung mit bekannten Parametern untersucht wird, die nicht zum Beispiel von vorzeitigen Wehen, vorzeitigem Blasensprung, Infektionen etc. und Ihren Konsequenzen für die weitere Fetalentwicklung betroffen sind. Frühgeburt beim Menschen als Indikation zur LRI muss im Hinblick auf die Interpretationen von Auswirkungen antenataler LRI mit GC berücksichtigt werden. Tiermodelle zeigen daher immer nur Teilaspekte auf.[312]

1.6 Zielsetzung

Die Behandlung mit GC während der Schwangerschaft stellt ein etabliertes Modell zu Untersuchung der Auswirkungen von maternalem Stress, als auch von Effekten der in der klinischen Routine angewandten LRI, auf die Fetalentwicklung und die Entstehung von langfristigen Störungen beim Kind dar. In diesem Zusammenhang kommt der Plazenta als Vermittler der maternalen GC-Wirkung eine besondere Bedeutung zu. Das Ausmaß der GC-induzierten Effekte hängt unter anderem von dem Zeitpunkt der Gabe während der Schwangerschaft, der Dosierung, dem Geschlecht als auch den kompensatorischen Fähigkeiten der Plazenta ab, auf diese „early life adversity" zu reagieren.

Im Folgenden werden daher sowohl tierexperimentelle Arbeiten als auch Studien am Menschen vorgestellt, deren Ziel es war, zum besseren Verständnis der Auswirkungen erhöhter GC-Exposition auf die Fetalentwicklung in Bezug auf den Behandlungszeitpunkt (Frühschwangerschaft, Spätschwangerschaft), die Dosierung, die Rolle der Plazenta, Interaktionen mit der HPA-Achse als auch mögliche geschlechtsspezifische Aspekte beizutragen.

Hierbei wurde übergreifend die Hypothese verfolgt, dass eine antenatale erhöhte GC-Exposition zu einer Beeinträchtigung der Fetalentwicklung mit Veränderung der fetalen und neonatalen Anthropometrie führen könnte. Die Veränderungen sind dabei sowohl dosis- als auch geschlechtsabhängig und der Plazenta kommt eine besondere Rolle in diesem Zusammenhang für Aspekte der fetalen Programmierung zu.

Übergreifendes Ziel der vorgestellten Studien war es, im Tierexperiment (Schaf) die Auswirkungen der antenatalen GC-Exposition in Bezug auf den Behandlungszeitraum in der Spätschwangerschaft, im paradigmatischen Sinne der antenatalen LRI und im Sinne eines maternalen Stresses in der Frühschwangerschaft zu untersuchen. Im Fokus standen hierbei die Untersuchung der Auswirkungen der antenatalen GC-Exposition auf das fetale Wachstum, die Entwicklung der fetalen HPA-Achse, Interaktionen mit der Plazenta und Untersuchungen der Plazenta selbst.

Im Sinne eines translationalen Ansatzes wurden darüber hinaus die Ergebnisse der tierexperimentellen Studien dann auch beim Menschen anhand umfangreicher, epidemiologisch-klinischer Untersuchungen zu Folgen antenataler GC-Behandlung überprüft, ihre Auswirkungen auf die Fetalentwicklung und die Plazenta untersucht und die Ergebnisse ebenfalls im Folgenden exemplarisch vorgestellt und diskutiert.

Alle Publikationen der vorliegenden Habilitationsschrift sind in internationalen Zeitschriften mit „peer reviews" veröffentlicht oder im Druck. Die vorgestellten Ergebnisse waren Gegenstand von Postern und Vorträgen auf nationalen und internationalen Kongressen. Die Arbeiten sind vollständig und der Reihe nach angefügt und werden im Anschluss ausführlich im Vergleich zur Literatur diskutiert.

2 Ausgewählte Originalarbeiten

2.1 Tierexperimentelle Arbeiten

2.1.1 Glukokortikoidexposition in der Spätschwangerschaft

T. Braun, S. Li, T. J. Moss, J. P. Newnham, J. R. Challis, P. D. Gluckman and D. M. Sloboda: "Maternal betamethasone administration reduces binucleate cell number and placental lactogen in sheep." J Endocrinol 2007; 194:337-347[98]

Hintergrund: Antenatale, maternale GC-Therapie bei drohender Frühgeburt stellt seit Ende der 70er Jahre das etablierte Therapieverfahren zur fetalen LRI mit Senkung neonataler Morbidität und Mortalität dar. Neben diesen positiven Effekten sind sowohl im Tiermodell als auch beim Menschen, insbesondere bei repetitiven GC-Gaben, eine Verringerung des Geburtsgewichts beschrieben worden. Im Schafmodell stellt das aus der Plazenta stammende ovine Plazentalaktogen (oPL) das entscheidende fetale Wachstumshormon dar. oPL wird durch spezialisierte, aus dem fetalen Trophoblasten stammenden binukleären Zellen (BNC) produziert und in die maternale und fetale Zirkulation sezerniert. oPL nimmt mammo-, lakto- und sommatotrop Einfluss auf den maternalen und fetalen Stoffwechsel. **Arbeitshypothese:** Die mit einer Wachstumsrestriktion einhergehenden GC-induzierten Effekte könnten durch die Plazenta vermittelt werden und ursächlich mit einer Beeinträchtigung der plazentaren oPL-Hormonproduktion einhergehen. **Ziel:** Untersuchung der Auswirkungen von antenataler GC-Exposition mit BET auf das fetale Wachstum und die Rolle von Plazentalaktogen beim Schaf. **Methode:** Trächtige Mutterschafe mit Einlingsschwangerschaften wurden randomisiert und mit Injektionen von Kochsalzlösung oder einer (104 Tagen Schwangerschaft; dG), zwei (104 und 111 dG) oder drei (104, 111 und 118 dG) Dosen BET (0,5 mg / kg) behandelt. Plazentagewebe sowie maternales und fetales Plasma wurden vor, während und nach dem Zeitraum der BET-Behandlung gesammelt. **Ergebnisse:** Bereits nach einer Gabe BET in klinisch relevanter Dosis war die Anzahl der BNC im Vergleich zu den Kontrollplazenten anhaltend deutlich verringert. Die Reduktion der BNC war mit einer Verringerung der oPL-Proteinlevel in der Plazenta sowie mit einer Verringerung der oPL-Hormonlevel im maternalen und fetalen Plasma assoziiert. **Diskussion/Schlussfolgerung:** Reduzierte oPL-Hormonspiegel könnten somit direkt oder über Regulation assoziierter Stoffwechselwege zu der mit GC-Gaben einhergehenden Geburtsgewichtsverringerung führen. Der Mechanismus, durch den GC die Funktion der BNC beeinflusst, ist weiterhin ungeklärt und Gegenstand weiterer Forschungsarbeiten.

Weitere eigene, relevante Publikationen zu diesem Thema:

T. Braun, S. Li, T.J.M. Moss, K.L. Connor, D.A. Doherty, I. Nitsos, J. P. Newnham, J.R.G. Challis and D. M. Sloboda: "Differential appearance of placentomes and expression of prostaglandin H synthase type 2 in placentome subtypes after betamethasone treatment of sheep late in gestation." Placenta 2011; 32:295-303[135]

J.R.G. Challis, K. Connor, S.G. Matthews, S. Lye, I. Caniggia, F. Petraglia, A. Imperatore, D. M. Sloboda, S. Li, **T. Braun**, W. Li, J. Newnham: "Development of the Fetal Hypothalamic-Pituitary-Adrenal-Placental Axis: Implications for Postnatal Health." Buchbeitrag in "In Early Life Origins of Human Health and Disease", Karger Verlag 2009; pp. 89-99[239]

Maternal betamethasone administration reduces binucleate cell number and placental lactogen in sheep

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Abstract

The placenta may mediate glucocorticoid-induced fetal growth restriction. Previous studies have examined effects of fetal cortisol in sheep, which reduces placental binucleate cell (BNC) number; the source of ovine placental lactogen (oPL). The effects of maternal GC are unknown. Therefore, this study examined the effects of maternal betamethasone (BET) administration on BNC number, distribution, placental oPL protein levels, and maternal and fetal plasma oPL levels. Pregnant ewes were randomized to receive injections of saline or one (104 days of gestation; dG), two (104 and 111 dG), or three (104, 111, and 118 dG) doses of BET (0·5 mg/kg). Placental tissue was collected before, during, and after the period of BET treatment. Fetal (121–146 dG) and placental (121 dG) weights were decreased after BET when compared with controls. In controls, the mean number of BNCs increased until 132 dG and decreased thereafter. Placental oPL protein levels peaked at 109 dG and remained stable thereafter. Maternal plasma oPL levels in controls increased across gestation; fetal plasma oPL levels decreased. BNCs were reduced by 24% to 47% after BET when compared with controls at all ages studied. Placental oPL protein levels, maternal, and fetal plasma oPL levels were also reduced after BET injections, but recovered to values that were not different to controls near term. BET disrupted the normal distribution of BNCs within the placentome. These data may suggest a placental role in growth restrictive effects of prenatal maternal BET exposure through alterations in placental output of oPL, a key metabolic hormone of pregnancy.

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Introduction

One of the most effective and important therapies in perinatal medicine to manage pregnant women at risk of early preterm birth is the administration of synthetic glucocorticoids. Their use however, when given repeatedly has been subject to ongoing controversy and uncertainty (Newnham *et al.* 2002). Inappropriate exposure of the developing fetus to maternal glucocorticoids has been proposed as a mechanism for fetal programming (Seckl 1997). Maternal injection of synthetic glucocorticoids results not only in improved preterm respiratory function (Liggins & Howie 1972, Jobe *et al.* 1998, Moss *et al.* 2001, 2003), but also in growth restriction, altered hypothalamus–pituitary–adrenal (HPA) function, and insulin resistance in the offspring (Moss *et al.* 2001, Bloomfield *et al.* 2003, Sloboda *et al.* 2005*a*,*b*, 2007). Reduced fetal weight and alterations in fetal HPA function may be mediated by effects on the placenta.

The placental hormone, placental lactogen, is a member of the growth hormone family and secreted in humans by the syncytiotrophoblast (Handwerger & Freemark 2000, Lacroix *et al.* 2002*b*). Placental lactogen is found in both the maternal and the fetal circulation (Gluckman *et al.* 1979), and during pregnancy, placental lactogen in humans may play an important role in the regulation of maternal carbohydrate, lipid, and protein metabolism (Handwerger & Freemark 2000). In the fetus, placental lactogen has been suggested to have a role in the regulation of fetal growth, but this effect may be indirect through alterations in the maternal metabolic environment, maternal placental nutrient transfer to the fetus or may be mediated through stimulation of insulin-like growth factor (IGF) release (Oliver *et al.* 1992, Schoknecht *et al.* 1996). Ovine placental lactogen (oPL), has a molecular mass of 22 kDa and is a nonglycosylated 198 amino-acid polypeptide (Warren *et al.* 1990). It is produced by binucleate cells (BNCs), which are formed from uninucleated cells in the fetal trophectoderm

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(Kappes *et al.* 1992). BNCs account for 10–20% of the cells of fetal trophectoderm (Wooding *et al.* 1993). After cell maturation and migration to the fetal–maternal–placental interface, BNCs fuse with the maternal epithelium (Lacroix *et al.* 2002*a*). The oPL containing granules are transferred across the fetal–maternal–placental interface and released into both the maternal and the fetal vasculature (Wooding 1992). In sheep, maternal serum oPL or cotyledonary oPL mRNA is associated with much of the variation in fetal weight during gestation (Kappes *et al.* 1992). The number of BNCs decline near term, concomitant with a fall in maternal plasma oPL concentration (Wooding 1992) and in parallel with the late gestational increase in fetal cortisol levels, and the initiation of parturition in sheep (Liggins 1976, Challis *et al.* 2000, 2005). Placenta models using surgical ligation of one of the two umbilical arteries to create placental insufficiency in sheep resulted in reduced circulating oPL hormone levels in the maternal circulation, but a concomitant increase in levels in the fetal circulation (Newnham *et al.* 1986). Direct fetal cortisol infusion during late gestation has been shown to decrease the number of BNCs in the fetal trophectoderm (Ward *et al.* 2002), but there are few data available regarding the effects of maternal injections of glucocorticoids on placental BNC distribution and function. Due to its potential role in regulating fetal growth and its inverse relationship with cortisol, we hypothesized that changes in placental lactogen may underlie glucocorticoid-induced fetal growth restriction. Therefore, we investigated the effects of maternal betamethasone (BET) administration on the numbers and distribution of placental BNCs, on placental oPL protein levels, and on maternal and fetal plasma oPL levels in sheep.

Materials and Methods

Animals and tissues

All experimental procedures were approved by the Animal Experimentation Ethics Committee of the University of Western Australia and/or the Western Australian Department of Agriculture.

Prenatal treatments

Pregnant Merino ewes (*Ovis aries*) bearing singleton male fetuses were studied to eliminate potential confounding effects of fetal sex on BET response. Animals were of known gestational age (day of mating=Day 0) were allocated randomly to receive maternal injections of saline or BET. All animals were injected intramuscularly with 150 mg medroxyprogesterone acetate (Depo Provera; Upjohn, Rydalmere, Australia) at 100 dG to reduce pregnancy losses due to subsequent glucocorticoid treatment as described previously (Moss *et al.* 2001, Sloboda *et al.* 2002). Pregnant animals received injections of saline ($n=30$) or one (at 104 dG), two (at 104 and 111 dG), or three (at 104, 111, and 118 dG) doses of BET. Maternal BET (Celestone Chronodose; Schering Plough, Baulkham Hills, Australia) injections were given intramuscularly in a dose of 0·5 mg/kg body weight; saline injections were of a comparable volume (5–6 ml).

Tissue collection

Placental tissue was collected prior to (75, 84, and 101 dG), during (109 and 116 dG), and after (121–122, 132–133, and 146–147 dG) BET administration. At 109 dG ($n=6$) ewes had received one dose of BET given at 104 dG. At 116 dG ($n=6$) ewes had received two doses of BET given at 104 and 111 dG. At 121 dG and the following time points ewes had received three doses of BET given at 104, 111, and 116 dG. Pregnant ewes were killed with a captive bolt and fetuses were delivered by Cesarean section, weighed, and killed by decapitation. The major fetal organs were removed, weighed and collected for use in other studies. Changes in organ weights were previously reported (Sloboda *et al.* 2005*a*). Maternal and fetal plasma samples were collected, hysterectomy was performed, and placentomes were dissected from the uterus. Tissues were either snap frozen in liquid nitrogen before storage at −80 °C, or fixed in 4% paraformaldehyde (Sigma Chemical Co.) according to standard procedures for future embedding in paraffin prior to sectioning

Immunohistochemical localization and quantification of BNCs

Immunohistochemical detection of BNCs was performed on 6 μm paraffin-embedded sections. Sagittal cross sections were taken in the middle of the placentomes. A monoclonal rabbit antibody against oPL (1:500 dilution, generously donated by Prof. A Gertler) was used (Sakal *et al.* 1997, Kann *et al.* 1999, Leibovich *et al.* 2000) with avidin–biotin–peroxidase reagents (Elite Vectastain ABC Kit, Vector Laboratories, Burlingame, CA, USA) as described previously (Jacobs *et al.* 1991). Tissue sections from each of the treatment and control groups were processed simultaneously to allow direct comparison between experiments. A tissue section from 146 dG placentome tissue was used as a positive control. Negative controls were as follows: i) the oPL-primary antibody was substituted either by antibody dilution buffer or nonimmune rabbit serum (1:500 dilution); ii) (Seron-Ferre *et al.* 1978) the peroxidase-labeled secondary link antibody (goat anti-rabbit immunoglobulin) was substituted with PBS (pH 7·5) wash buffer; and iii) the slide section was only incubated with PBS (pH 7·5) diluent before the addition of the substrate–chromogen solution.

Image analysis

Semi quantitative analyses were performed using computerized image analysis (ImagePro Plus 4.5, Media Cybernetics, Silver Spring, MD, USA). To reduce the number of false positive counts, BNCs were counted only if >30% of the cytoplasm of a BNC was visible and at least 80% of the randomly selected field of view was covered with placental tissue (Ward *et al.* 2002). BNC localization and distribution

was analyzed within three levels, which refer to previously reported zones in a placentome (Burton *et al.* 1976). L1 contained 1, 6, 7, and 8 fields of interest and was taken in the zona intima, closely to the capsule of the placentome. L2 contained 2, 5, 8, and 11 fields of interest and refers best to the intermediate zone of the placentome. L3 contained 3, 4, 9, and 10 fields of interest and refers best to the hemophagus zone, closely taken to the chorion-allantois (Fig. 1). A total of 12 random fields of view (area 750 μm^2) were counted per section of immunostained tissue at a magnification of 20×. Two animals for each gestational age and study group were randomly selected. At least three placentomes per animal, regardless of placentome subtype, were immunostained and at least four sections of immunostained tissue were counted per placentome. There were two study groups and eight gestational age time points, containing a total of 78 animals with over 3700 fields of interest counted.

Quantification of placental oPL protein levels: western blotting

Frozen placentome samples ($n=140$ from 78 sheep) were homogenized on ice for 1 min in radioimmuno precipitation assay (RIPA) lysis buffer (50 nM Tris–HCl (pH 7·5 Sigma Chemical Co), 150 mM NaCl (EMD, Gibbstown, NJ, USA), 1% w/v sodium deoxycholatic acid (Sigma Chemical Co.); 0·1% SDS (Bio-Rad), 100 mM sodium orthovanadate (Sigma Chemical Co.), 1% (vol/vol) Triton X-100 (Fisher Scientific, Ottawa, Canada), and Complete MiniEDTA-free protease inhibitors (Roche Molecular Biochemicals). Homogenates were centrifuged at 4 °C at 15 000 *g* for 15 min and supernatants were collected. Protein concentrations were determined using the Bradford assay (Bradford 1976). Proteins were separated by electrophoresis on 12% polyacrylamide gels at 80 V at 4 °C. Samples from control and treatment groups, from each gestational age, were run together on one gel to allow comparisons between groups. Separate runs were done with randomly picked controls or treated placentomes to allow comparisons across gestation. Each blot was repeated at least three times. Separated proteins were transferred onto nitrocellulose membranes (Bio-Rad Laboratories). The resultant blots were stained with S-Ponceau (0·1% w/v Ponceau S in 5% acetic acid v/v; Sigma Chemical Co.) to verify equal loading and transfer. The blots were washed with PBS and 0·1% Tween-20 (PBS-T; Sigma Chemical Co.) and incubated for at least 2 h at 4 °C in blocking solution (5% skim milk powder w/v in PBS) on a mechanical shaker to block nonspecific binding. Membranes were incubated with the same primary monoclonal rabbit antibody against oPL as used for immunohistochemistry, but at a dilution in the ratio of 1:1000 with blocking solution (5% skim milk powder in PBS) for 1 h. All blots were then rinsed six times for 5 min each with PBS-T and incubated with secondary antibodies conjugated to horseradish peroxidase (anti-rabbit IgG-horseradish peroxidase; Amersham Life Sciences) dilution in the ratio of 1:1000 in blocking solution (5% skim milk powder in PBS) for 1 h. Blots were washed (5 min×6) and the antibody–antigen complex was detected using a chemiluminescence detection system enhanced chemiluminescence (ECL), Perkin–Elmer, Waltham, MA, USA). Membranes were incubated without primary antibody for negative controls. Blots were exposed to autoradiographic film (Eastman Kodak X-Omat) for visualization. oPL was identified as two close bands of 22 kDa (Fig. 6). All blots were reincubated with anti ß-actin (Sigma Chemical Co.) as an internal control to allow for corrections in

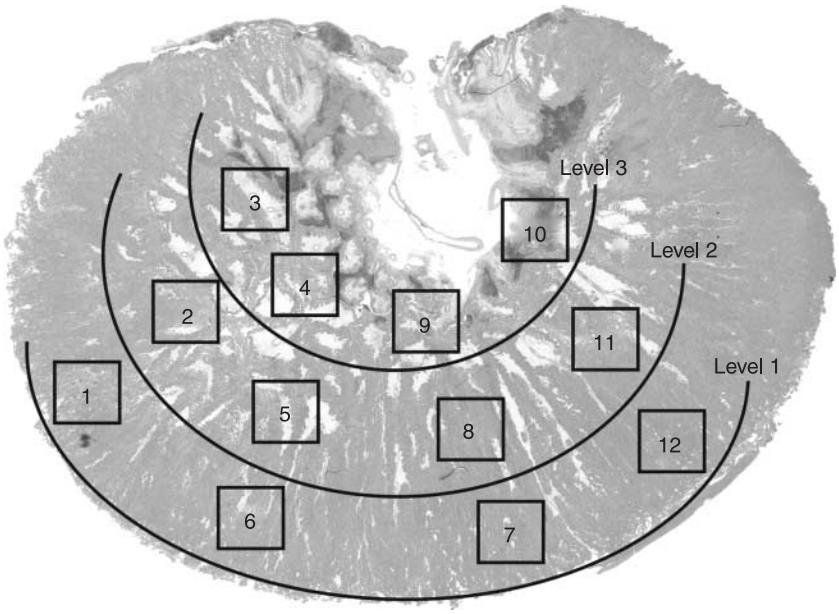

Figure 1 Macroscopic view of a hamatoxylin stained placentome. A total of 12 random fields of view, grouped into three levels were counted per section of immunostained tissue.

gel loading and transfer. Band density for both the protein of interest and ß-actin was quantified by densitometry using Scion Image Analysis software Alpha 4.0.3.2 (Scion, Maryland, USA). The results were expressed as the ratio of protein to ß-actin as relative optical density (ROD) units.

Quantification of oPL plasma levels: RIA

Plasma oPL concentrations were measured using equilibrium RIA previously described and validated in sheep (Oliver *et al.* 1992). The minimal detectable dose was 0·2 ng/ml, the intraassay coefficient of variation was 4–6%, and the interassay coefficient of variation was 7·92%. Values are expressed in terms of recombinant oPL (M3RD86, GenTech; Arcade, JY, USA).

Statistical analysis

Statistical analysis was performed using SigmaStat Statistical Software (SigmaStat v2.03; SYSTAT Software Inc., Chicago, IL, USA). Data were first analyzed for normality and equal variance (Kolmogorov–Smirnov test). Data that were not normally distributed were log transformed to achieve normality. In control placentomes, BNC numbers, oPL protein levels, and plasma oPL hormone levels were analyzed across gestation (75–146 dG) using a one-way ANOVA (Tukey), where gestational age was the factor. In treatment groups (109, 116, 121, 132, and 146 dG) BNC numbers, placental oPL protein levels and plasma oPL levels were analyzed using a two-way ANOVA (Tukey), where gestational age and treatment were factors. The number of BNCs and the distribution (total numbers and levels L1–L3) were analyzed separately for each gestational age (109, 116, 121, 132, and 146 dG) using a two-way ANOVA (Tukey), where level and treatment were the factors. The relationship between the number of BNCs, placental oPL protein levels or plasma oPL levels with fetal and placental weights was assessed by linear regression, desired power for the performed test was accepted for values $\alpha 0{\cdot}800$. Data are presented as mean$\pm$S.E.M. In all cases, statistical significance was accepted for values $P<0{\cdot}05$.

Results

Fetal and placental weight

Fetal weight increased across gestational age in control ($P<0{\cdot}001$) and BET-treated animals ($P<0{\cdot}001$; Fig. 2). After maternal BET treatment at 104, 111, and 118 dG, fetal weight was significantly reduced at 121, 132, and 146 dG when compared with control animals ($P<0{\cdot}05$). There was a significant decrease in total placental weight in control animals between 75 and 146 dG ($P<0{\cdot}05$). Total placental weight was decreased at 121 dG when compared with controls after BET administration ($P<0{\cdot}05$; Fig. 2).

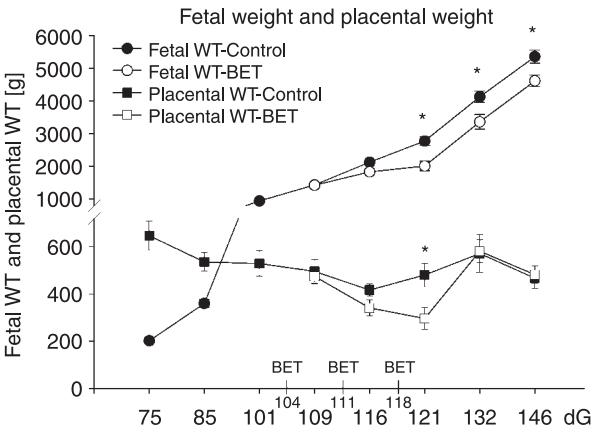

Figure 2 Fetal weight and placental weight across gestation. Fetal weights are represented by circles (black, control; open, BET). Placenta weights are represented by squares (black, control; open, BET). (Significance level set as $P<0{\cdot}05$; *$P<0{\cdot}05$.)

Binucleate cells

The effect of BET on BNC number To investigate the effect of BET on BNCs numbers and distribution, immunohistochemistry was used to stain the BNCs with anti-oPL antibody. BNCs were identified as oPL positive cells within tissue sections as oPL localized predominately to the BNCs, with little immunostaining in the fetal syncytium (Fig. 3; Handwerger *et al.* 1977, Kappes *et al.* 1992, Ward *et al.* 2002). In control animals, the overall mean number of BNCs increased across gestation from 75 to 109 dG ($P<0{\cdot}05$) with peak BNC numbers at 132 dG ($P<0{\cdot}05$; Fig. 4). From 132 to 146 dG, the BNC numbers in controls decreased to values similar to those observed at 109 and 116 dG ($P<0{\cdot}05$). BET administration resulted in a significant reduction in BNCs between 109 and 146 dG to values that were 24–47% lower than in control animals ($P<0{\cdot}001$; Fig. 4). The number of BNCs at 132 dG was significantly greater than at earlier time points in pregnancy, or in BET-treated animals at term ($P<0{\cdot}05$; Fig. 4).

The effect of BET on BNC localization and distribution The localization and distribution of BNCs in placentomes is shown in Fig. 5. In controls, the distribution of BNCs at 109 was similar between the three levels. At later time points (121 and 132 dG), BNCs were more numerous in L2 when compared with L1/L3 ($P<0{\cdot}05$). Near term the mean number of BNCs in controls were highest in L1 when compared with L3 ($P<0{\cdot}001$). A decrease in BNC number after BET exposure was observed within all three levels at each gestational age studied. At 109, 116, 121, and 146 dG, the number of BNCs at L3 was significantly lower than at L1/L2 ($P<0{\cdot}05$; Fig. 5). The coefficient of variance (CV) per animal for the whole placentome (fields 1–12) was 19·21% at 75–101 dG, 12·11%-control versus 23·93%-BET at 109–121 dG ($P<0{\cdot}05$), and 14·88%-control versus 12·85%-BET at 132–146 dG. The significant differences in CV between controls and BET regarding the levels studied at 109–121 dG

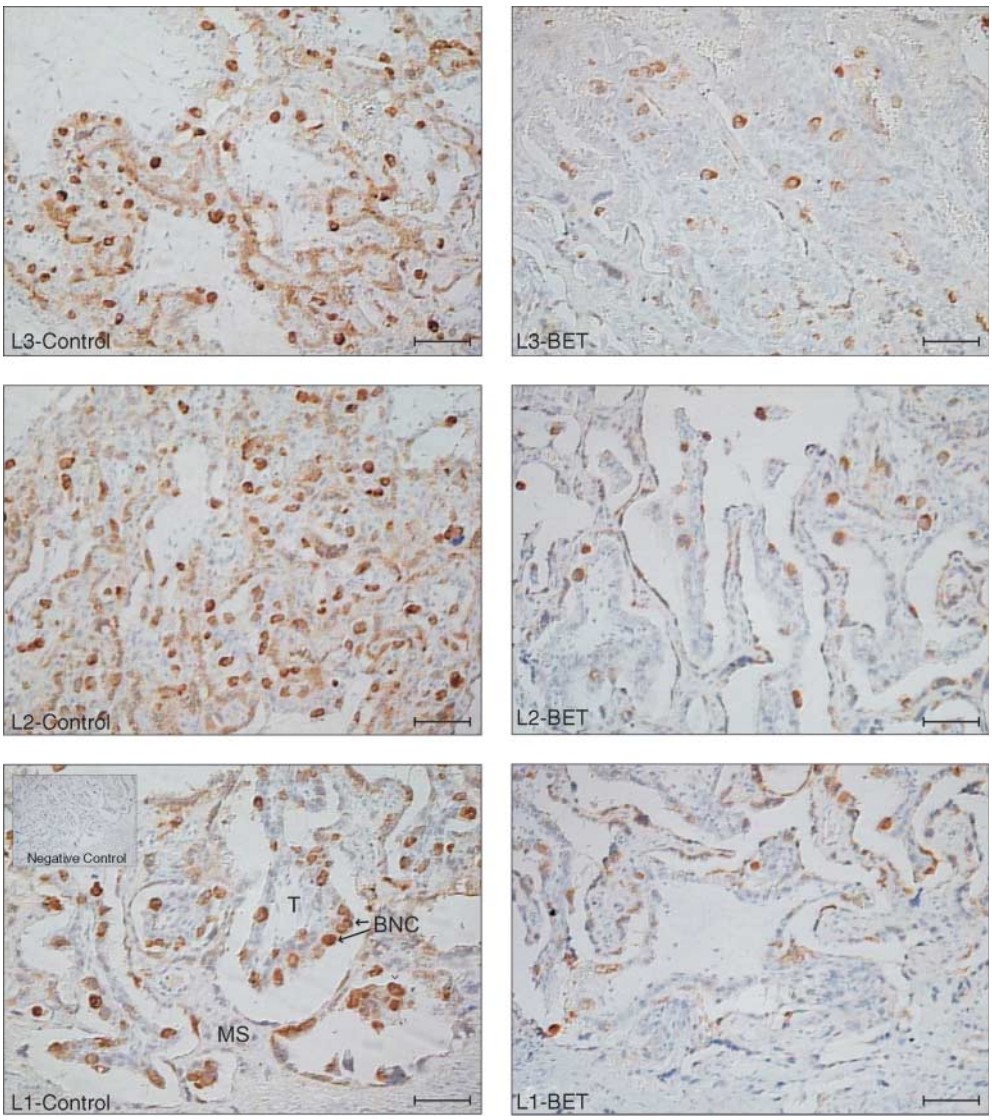

Figure 3 Representative photographs of binucleate cell staining in placentomes at 116 dG in control (left panels) and betamethasone (right panels) animals. Sections were taken at L1, L2, and L3 of the placentome (Fig. 1). Negative control (insert). T, trophoblast; MS, maternal syncytium; BNC, binucleate cell. (Scale bar represents 2·5 μm.)

(control: L1 = 7·54%★, L2 = 8·53%★, and L3 = 11·83%; and BET: L1 = 13·39%★, L2 = 15·36%★, and L3 = 20·97%; ★$P<0·05$).

Placental oPL protein levels

Placental oPL protein was identified as two close bands of 22 kDa with no other background signal (Fig. 6). In control animals, the overall oPL protein levels increased from 75 to 109 dG ($P<0·05$), followed by a decrease from 109 to 116 dG ($P<0·05$; Fig. 7). There was no significant further change in protein levels from 116 to 146 dG. In BET-treated animals, placental oPL protein levels were 52–72% lower than in control animals at 116 and 121 dG ($P<0·05$; Fig. 7). There were no significant differences in placental oPL protein levels between control and BET groups thereafter (121–146 dG; Fig. 7).

Maternal and fetal circulating oPL plasma levels

Maternal plasma oPL levels in controls significantly increased across gestation ($P<0·05$; Fig. 8). Fetal plasma oPL levels in controls significantly decreased near term (146 dG versus 101 and 75 dG, $P<0·05$; Fig. 8). Maternal and fetal plasma oPL levels were significantly decreased when compared with controls following BET injections (109, 116, and 121 dG, $P<0·05$; Fig. 8). Near term, maternal, and fetal plasma oPL levels in BET recovered to values that were not significantly different from controls (Fig. 8).

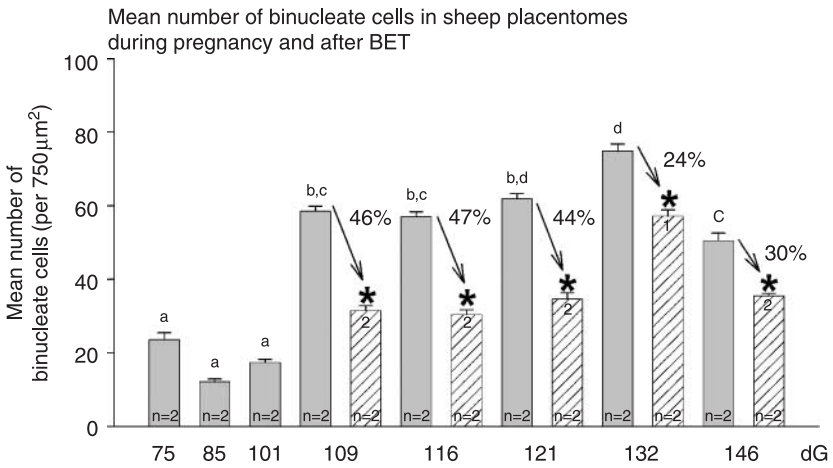

Figure 4 Mean number of binucleate cells in sheep placentomes during pregnancy and after BET. Grey bars are control placentomes, white coarse bars are BET placentomes. Significant differences in BNC numbers between control and BET placentomes are represented by stars. Different letters on the histogram represent significant differences in control groups over gestation. Different numbers on the histogram represent significant differences in BET groups over gestation. (*n*, number of animals studied; significance level set as $P<0{\cdot}05$; *$P<0{\cdot}001$.)

The relationship between BNC number, oPL protein level, plasma oPL levels with fetal weight and placental weight

The number of BNCs was positively associated with fetal weight in controls, but not in BET-treated animals (Table 1). There was no significant association between BNCs and oPL protein levels, placenta weight or fetal circulating plasma oPL levels either in controls or in BETs. Maternal circulating oPL levels showed a positive association with the number of BNCs in BETs, which was not seen in the controls.

In control and BET-treated animals, oPL protein levels were positively associated with maternal circulating oPL plasma levels (75–146 dG) and fetal weight (75–109 dG;

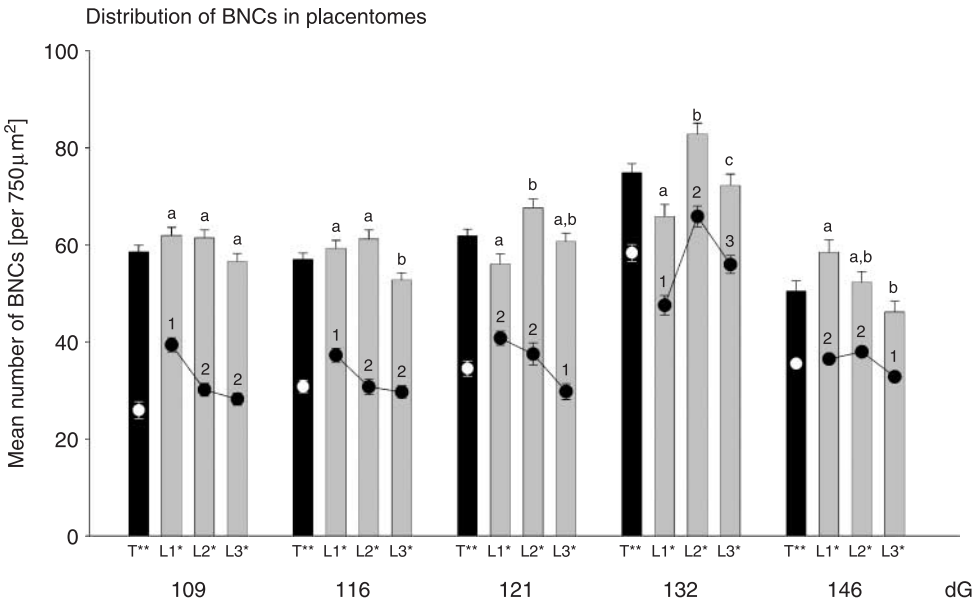

Figure 5 Distribution of mean number of BNCs in different levels of the placentome. Grey bars are control, black dots are BET placentomes. Black bars and white dots represent total number of BNCs (T). At each gestational age two randomly selected animals were studied. Different letters on the histogram represent significant differences in control groups at each gestational age. Different numbers on the histogram represent significant differences in BET groups at each gestational age. (Significance level set as $P<0{\cdot}05$; *control versus BET in L1 to L3 $P<0{\cdot}001$; **control versus BET with $P<0{\cdot}001$.)

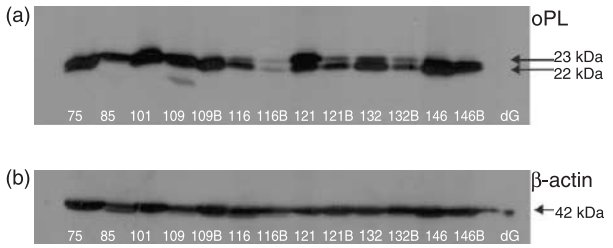

Figure 6 Representative western blots of ovine placental lactogen (a, ~22 kDa) and ß-actin (b, ~42 kDa) across gestation (dG) in control- and betamethasone (B)-treated animals.

Table 1). After BET treatment there was an additional positive association between oPL protein levels and fetal circulating plasma oPL levels and placenta weight (Table 1).

In control sheep, fetal weight was inversely associated with placental weight early to mid-gestation (75–146 dG) and positively associated with late in gestation (109–146 dG). There was no association between placental weight and maternal circulating plasma oPL level, but fetal circulating plasma oPL level was positively associated with controls and BETs (Table 1). Fetal weight presented a strong association to maternal circulating plasma oPL levels across gestation in either controls or BETs (Table 1).

Discussion

Antenatal glucocorticoid administration has beneficial clinical effects for preterm infants. There are, however, a number of known deleterious effects with repeated prenatal glucocorticoid exposure. This study, to our knowledge, is the first study to show

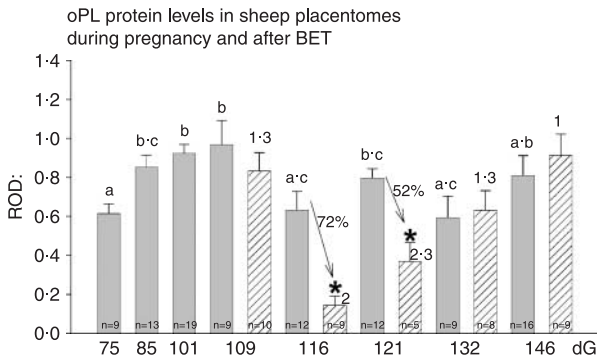

Figure 7 Ovine placental lactogen protein levels in sheep placentomes during pregnancy and after BET. Grey bars are control, white coarse bars are BET placentomes. Significant differences in oPL protein levels between control and BET placentomes are represented by stars. Different letters on the histogram represent significant differences in control groups. Different numbers on the histogram represent significant differences between groups. (*n*, numbers of placentomes analyzed; significance level set as $P<0{\cdot}05$; $^*P<0{\cdot}05$.)

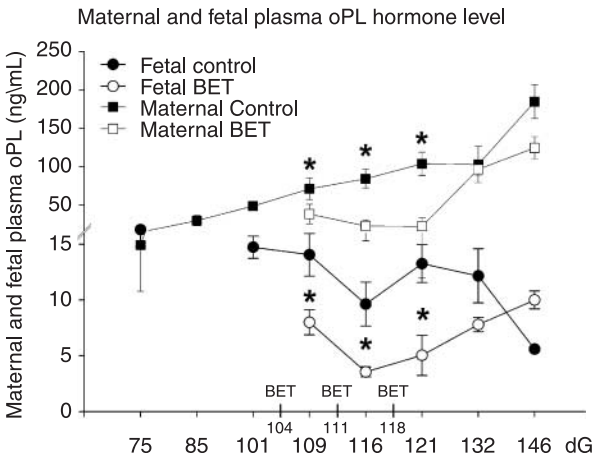

Figure 8 Maternal and fetal plasma oPL hormone level across gestation. Maternal plasma hormone levels are represented by squares (black, control; open, BET). Fetal plasma hormone levels are represented by circles (black, control; open, BET). (Significance level set as $P<0{\cdot}05$; $^*P<0{\cdot}05$.)

that repeated maternal i.m. injections of BET in the last third of gestation reduced placental BNC numbers, decreased oPL protein levels, and decreased circulating oPL levels in the mother and the fetus. We have demonstrated that BNC number, placental oPL protein levels, and circulating maternal and fetal oPL levels were positively associated with fetal weight. Furthermore, we have shown that maternal BET administration changed the distribution and localization of BNCs within the placentomes. Based on previous observations, we suggest that the demonstrated growth restricting effects of maternal BET (Johnson *et al.* 1981, Jobe *et al.* 1998, Sloboda *et al.* 2000) may be associated with our observed alterations in BNC formation and placental production of oPL.

In contrast to the human placenta, ruminant (such as sheep) placentae are synepitheliochorial and demonstrate noninvasive placentation (Handwerger *et al.* 1975). Although there are differences in morphology and in the ultrastructure of the placenta between humans and sheep, studies comparing placental vasculature between sheep and humans report that the sheep placenta in many aspects is workable as a model for the human placenta (Leiser *et al.* 1997). Placental lactogen is found in humans and sheep, but produced by somewhat different trophoblast types. Early studies in human pregnancies did not demonstrate significant changes in maternal human placental lactogen levels after glucocorticoid treatment (Maltau *et al.* 1979, Socias *et al.* 1979), others observed a significant reduction in maternal human placental lactogen after maternal dexamethasone treatment (Lange & Anthonsen 1980).

With increasing gestational age changes in placenta gross morphology and ultrastructure occur as adaptations in response to nutritional and endocrine challenges (Sibley *et al.* 2005). oPL is somatogenic; stimulating ornithine decarboxylsae activity, amino acid uptake, glycogen synthesis, and IGF secretion (Anthony *et al.* 1995). During pregnancy,

Table 1 The relationship (expressed as *r*-values) between binucleate cell (BNC) number, ovine placental lactogen (oPL) protein level, plasma oPL levels with fetal weight and placental weight

		No. of BNCs	oPL protein	Fetal plasma oPL	Maternal plasma oPL	Fetal weight
oPL protein						
75–146 dG	Control	−0·300		+0·253	0·000166	−0·192
	BET	+0·519		+0·683*	+0·640*	+0·329
75–109 dG	Control	−0·063		−0·281	+0·448[a]	+0·564*
109–46 dG	Control	+0·281		−0·137	−0·163	−0·331
Days 109 and 116	Control	+0·165		+0·402	0·0653	−0·653[a]
	BET	−0·0917		+0·927*	+0·552	−0·535
Days 116 and 121	Control	+0·102		+0·522	+0·461	+0·448
	BET	+0·771		+0·197	0·0305	+0·268
Fetal plasma oPL						
75–146 dG	Control	−0·271	+0·253		0·0428	−0·432*
	BET	+0·240	+0·683*		+0·568*	+0·303
75–109 dG	Control	0·0253	−0·281		+0·427	−0·370
109–146 dG	Control	−0·130	−0·137		+0·219	0·0217
Maternal plasma oPL						
75–146 dG	Control	+0·507[a]	0·00016	0·0428		+0·849*
	BET	+0·610*	+0·640*	+0·568*		+0·838*
75–109 dG	Control	+0·353	+0·448*	+0·427		+0·699*
109–146 dG	Control	+0·112	−0·163	+0·219		+0·678*
Fetal weight						
75–146 dG	Control	+0·775*	−0·192	−0·432*	+0·838*	
	BET	+0·464	+0·329	+0·303	+0·838*	
75–109 dG	Control:	+0·730*	+0·564*	−0·370	+0·699*	
109–146 dG	Control	−0·0190	−0·331	0·0217	+0·678*	
Days 116–146	Control	+0·0748	−0·248	+0·132	+0·838*	
	BET	+0·545	+0·835*	+0·660[a]	+0·919*	
Placental weight						
75–146 dG	Control	−0·213	+0·0518	+0·502*	0·0299	−0·298*
	BET	+0·560[a]	+0·597*	+0·582*	+0·542[a]	+0·326[a]
75–109 dG	Control	−0·182	−0·337	+0·372	−0·235	+0·225
109–146 dG	Control	−0·299	+0·125	+0·144	+0·291	+0·397*

Linear regression between control- and betamethasone-treated animals. Significance level set as $P<0·05$; *$P<0·05$.
[a]The power of the performed test is below the desired power of 0·800.

oPL stimulates development of the mammary glands (Ward *et al.* 2002) and redirects substrate utilization to make glucose available for transport to the fetus (Breier *et al.* 1994). oPL is produced by the BNCs and is found in both the maternal and the fetal circulation (Gluckman *et al.* 1979). It is well established that maternal stress (changes in nutrition or glucocorticoid administration) during pregnancy can restrict fetal growth and induce adult disease. Changes in gross morphology and ultrastructure of the placenta are interrelated and lead to alterations in surface area, vascularity, barrier thickness, and cell composition of the placenta, all of which influence placental transport characteristics (Sibley *et al.* 1997). It is therefore likely that altered placental morphology, growth and/or function underpin the changes in fetal growth and increased disease risk in models of prenatal undernutrition, and/or prenatal glucocorticoid exposure. We have demonstrated that the number of BNCs in control animals increased from early (75 dG) to late (132 dG) gestation and subsequently declined to term (146 dG). The late gestational BNC decrease is most likely associated with rising fetal circulating cortisol levels at this time (Wooding *et al.* 1993, Fowden *et al.* 1998, Ward *et al.* 2002). It has been reported previously that cortisol infusion, directly into the fetus during late gestation decreased BNC number in fetal trophoectoderm (Ward *et al.* 2002). Further, when the natural cortisol surge at the end of gestation was abolished by fetal adrenalectomy or fetal hypophysectomy, the normal prepartum decline in BNC numbers was not observed (Ward *et al.* 2002). We now demonstrate in the present study that administration of synthetic glucocorticoids also reduces placental BNC numbers. Previous studies suggest that chorionic BNCs deliver their products to the maternal and fetal circulation by migrating to and fusing with the fetomaternal syncytium (Wooding *et al.* 1992). Intrafetal cortisol injections have been shown to reduce BNC numbers by either increasing the rate of BNC migration across the fetal–maternal interface or inhibition of BNC formation (Ward *et al.* 2002). In our study, BET administration may have prevented the natural occurring increase in BNC numbers observed from early (75 dG) to mid-gestation (109 dG) in controls and may be indicative of inhibited BNC formation.

We have shown for the first time, that BNCs are differentially distributed throughout different areas of an ovine placentomes and that BET administration significantly

altered this distribution. This has clear implication for placental function. Placenta nutrient transport has long been known to be dependent on vascular development. Placental vascularity in sheep during pregnancy occurs through an increase in the number and surface density of capillaries, particularly on the fetal side of the placentome (Stegeman 1974, Reynolds *et al.* 2005). Placental surface area for nutrient exchange increases through elongation and increased branching of the fetal villi (Stegeman 1974, Macdonald & Fowden 1997). In sheep, BNCs from the fetal chorionic epithelium migrate through the apical tight junction of the fetal trophectoderm and fuse into the syncytium (Wooding *et al.* 1981). The observation of BNCs at all levels of the placentome suggests that the BNC population is continually developing in association with the expanding villous structure. The changes in BNC numbers with advancing gestation in L1 might be related to terminal villi development in the placentome, although this would not completely explain the higher values in L2 when compared with L1 at 121 and 132 dG. Furthermore, our observed BET induced reduction in BNCs in the superior portions of the placentomes (L2 and L3) at time points immediately following BET injections (109, 116, and 121 dG) suggests that the terminal villi development with new BNCs in L1 could explain why there are fewer numbers of BNCs in BET-treated animals in L3 when compared with L1. It also appears that placenta has the capacity to recover, given that this effect diminished later in gestation (132 and 146dG). Others have speculated that the reduction in BNCs caused by cortisol exposure influences the expansion of the fetomaternal syncitium, barrier thickness, and placental hormone secretion (Ward *et al.* 2002, Fowden *et al.* 2006). Consistent with this, placental growth impairment through undernutrition in guinea pigs resulted in a reduction surface area by 60–70% and an increase in barrier thickness between maternal and fetal capillaries by 40% late in gestation (Roberts *et al.* 2001). We speculate that maternal administration of BET might influence maternal–fetal nutrient transfer by impairment of placental surface area and barrier thickness, and have downstream effects on fetal growth. It is clear that a full understanding of this process using accurate stereological and functional investigations needs to be done.

Previous studies suggest that oPL may play an important role in fetal growth through its actions on maternal metabolism regulating fetal substrate availability (Handwerger *et al.* 1975). Although some previous studies reported higher values for maternal and fetal plasma oPL, Bauer *et al.* (1995) reported similar values to ours. The fact that maternal plasma oPL levels significantly increased with advancing gestation in control animals is not dissimilar from previous findings (Chan *et al.* 1978). oPL is detectable in maternal serum as early as 50 dG (Handwerger *et al.* 1977). Near term, the decline in BNC number is coincident with a decline of maternal serum oPL concentrations (Handwerger *et al.* 1977, Taylor *et al.* 1980, Wooding *et al.* 1993). Our data on placental oPL protein levels permit us to relate the effects of BET on BNC number to their function. We have shown that BET exposure significantly decreased the number of BNCs from 109 to 146 dG and decreased overall placental oPL protein levels and fetal and maternal plasma oPL levels. At 132–146 dG placental oPL protein levels and fetal and maternal oPL plasma levels returned back to values that were not different from controls even though BNC number remained low. This is suggestive of fewer BNCs producing more oPL and we speculate that this increased oPL protein output may reflect a 'functional adaptation' of BNCs in BET exposed placentae. The exact mechanism by which glucocorticoids act on BNCs and regulate the placental lactogen secretion requires further investigation. The variation in fetal weight has been attributed to placental weight, maternal serum oPL, and cotyledonary oPL mRNA concentrations (Kappes *et al.* 1992, Fowden *et al.* 2006). In the present study fetal weight was positively correlated with maternal oPL levels and after maternal BET administration, maternal oPL levels decreased. Placental weight was not associated with maternal oPL plasma levels, but showed a positive association with fetal plasma oPL levels (75–146 dG) and with fetal weight (109–146 dG). The lack of association between fetal weight and fetal plasma oPL suggests that BET has potentially disrupted the normal relationship between the maternal–fetal–placental unit at mid-gestation and we can speculate that the growth restricting effects of BET exposure could be associated with altered substrate supply to the fetus (Handwerger *et al.* 1975).

The exact mechanisms by which glucocorticoids influence placental and fetal growth remain unclear and requires further investigation. However, our current data provide some understanding into the potential role of the placenta. Previous studies suggest a decrease in placental transport capacity through an increase in the barrier thickness between fetal and maternal capillaries, and a decrease in placental surface area (Fowden *et al.* 2006). Our data may suggest a role of prenatal BET exposure in inducing changes in placental ultrastructure. Furthermore the growth restricting effects of synthetic glucocorticoid exposure could be mediated by direct effects of placental lactogens on maternal and fetal growth factor secretion and/or function. Studies in rats have suggested that the link between placental lactogen and growth could be achieved via IGFs (Karabulut et al.*et al.* 2001). Glucocorticoids have been shown to increase maternal serum IGF-I levels and a single course of antenatal BET resulted in a decrease of fetal serum IGF-II levels (Ahmad et al.*et al.* 2006). A thorough understanding of how placental lactogens and other placental hormones influence fetal growth and fetal development will provide a better understanding about the role of the placenta in the fetal adaptive response.

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2.1.2 Glukokortikoidexposition in der Frühschwangerschaft

T. Braun, S. Li, D.M. Sloboda, Wei L., M. Audette, T.M.J. Moss, G. Polglase, I. Nitsos, S.G. Matthews, J. P. Newnham, J. R.G. Challis: "Effects of maternal dexamethasone treatment in early pregnancy on pituitary-adrenal axis in sheep." Endocrinology 2009 150:5466-5477[136]

Hintergrund: Bei angeborenem androgenitalen Syndrom wird bereits in der Frühschwangerschaft täglich mit DEX therapiert um die Virilisierung eines weiblichen Feten zu verhindern. Diese Behandlung könnte zu langfristigen endokrinologisch-neurologischen Nebenwirkungen führen. **Arbeitshypothese:** Wir postulierten, dass die frühe maternale DEX-Therapie zum Zeitpunkt der Ausbildung des hypothalamisch-hypophysären Systems beim Schaf mit einer langfristigen Veränderung und Beeinträchtigung der Entwicklung desselben einhergeht. **Ziel**: Untersuchungen zu den unmittelbaren und langfristigen Auswirkungen der mütterlichen DEX-Therapie in der Frühschwangerschaft auf das fetale Wachstum und die Hypophysen-Nebennieren-Aktivität beim Schaf. **Methode:** Trächtige Mutterschafe mit Einlingsschwangerschaften wurden randomisiert und mit Injektionen von Kochsalzlösung (2ml, n=61) oder DEX (4x0,14mg/kg Mutterschafgewicht) am Tag 40 bis 41 der Schwangerschaft (dG) behandelt. An den Tagen 50, 100, 125 und 140 dG wurden fetales Plasma und Gewebe gesammelt. **Ergebnisse:** Bereits eine zweitägige maternale DEX-Therapie in der Frühschwangerschaft beim Schaf führt neben Geburtsgewichstveränderungen zu profunden, langfristigen und geschlechtsspezifischer Beeinträchtigung der fetalen Stressachse. 100 Tage nach erfolgter DEX-Therapie, gegen Ende des dritten Trimenons, finden sich bei männlichen und weiblichen Feten signifikant erhöhte Plasma Kortisol-Werte. Ursächlich fand sich bei den männlichen Feten eine Erhöhung der mRNA-Expression für die Schlüssenenzmye P450C17 und 3ßHSD der Kortisol-Biosynthese in der fetalen Nebennierenrinde. Diese ließen sich jedoch bei den weiblichen Feten nicht nachweisen. Ein maternaler Kortisol-Transfer via Plazenta konnte ausgeschlossen werden. **Diskussion/Schlussfolgerung:** GC-Therapie in der Frühschwangerschaft führt zu langfristigen, geschlechtsspezifischen Veränderungen in der HPA-Stressachse beim Schaf. Bei den weiblichen Feten wird diese durch eine vermehrte Nebennierenrinden-Kortisol-Synthese verursacht.

Weitere eigene, relevante Publikationen zu diesem Thema:

S. Li, D.M. Sloboda, T.J.M. Moss, I. Nitsos, G.R. Polglase, D.A. Doherty, J.P. Newnham, J.R.G. Challis, **T. Braun:** "Effects of glucocorticoid treatment given in early or late gestation on growth and development in sheep" JDOHaD 2013; 4(2): 146–156[322]

S. Li, I. Nitsos, G.R. Polglase, **T. Braun**, T.J.M. Moss, J.P. Newnham and J.R.G. Challis: "The effects of dexamethasone treatment in early gestation on hypothalamic-pituitary adrenal responses and gene expression at seven months postnatal age in sheep". Reprod Sci 2012;19:260-70[138]

H. Xu, D.M. Sloboda, L. Ehrlich, S. Li, J.W. Dudenhausen, J.P. Newnham, A. Plagemann, J.R.G. Challis and **T. Braun**: "The dilution effect and the importance of selecting the right internal control genes for RT-qPCR: a paradigmatic approach in fetal sheep" BMC Res Notes 2015; 8:58[323]

Effects of Maternal Dexamethasone Treatment in Early Pregnancy on Pituitary-Adrenal Axis in Fetal Sheep

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Fetal exposure to elevated levels of bioactive glucocorticoids early in gestation, as in suspected cases of congenital adrenal hyperplasia, may result in adverse neurological events. Fetal hypothalamic-pituitary-adrenal development and function may be involved. We investigated immediate and long-term effects of maternal dexamethasone (DEX) administration early in pregnancy on fetal growth and pituitary-adrenal activity in sheep. Pregnant ewes carrying singleton fetuses (total n = 119) were randomized to control (2 ml saline/ewe) or DEX-treated groups (im injections of 0.14 mg/kg ewe weight · 12 h) at 40–41 d gestation (dG). At 50, 100, 125, and 140 dG, fetal plasma and tissues were collected. DEX-exposed fetuses were lighter than controls at 100 dG ($P < 0.05$) but not at any other times. Fetal plasma ACTH levels and pituitary POMC and PC-1 mRNA levels were similar between groups. Fetal plasma cortisol levels were significantly reduced after DEX exposure in both male and female fetuses at 50 dG ($P < 0.05$), were similar at 100 and 125 dG, but were significantly higher than controls at 140 dG. At 140 dG, there was increased adrenal $P450C_{17}$ and 3β-HSD mRNA in female fetuses and reduced expression of ACTH-R mRNA in males. Fetal hepatic CBG mRNA levels mimicked plasma cortisol patterns. DEX exposure reduced CBG only in males at 50 dG ($P < 0.05$). Placental mRNA levels of 11β-HSD2 were increased after DEX in males ($P < 0.05$). Therefore, in sheep, early DEX may alter the developmental trajectory of the fetal hypothalamic-pituitary-adrenal axis, directly increasing fetal adrenal activation but not anterior pituitary function. In females, this effect may be attributed, in part, to increased fetal adrenal steroidogenic activity. **(*Endocrinology* 150: 5466–5477, 2009)**

Maternal administration of synthetic glucocorticoids (GC) (1) is an important clinical tool used both in the management of women at risk of early preterm birth and also in suspected cases of congenital adrenal hyperplasia. It has succeeded in reducing neonatal mortality and morbidity from respiratory distress syndrome and protects female fetuses from virilization (2–8). Experimental data from animal studies and observations of adverse medical events in human newborns have raised concerns about the safety of GC treatment and the impact it may have on fetal development. In rats, low-dose dexamethasone (DEX) given during pregnancy modestly reduces birth weight but does not affect litter size, although it causes later hypertension, hyperglycemia, and hyperinsulinemia. There is increased hypothalamic-pituitary adrenal (HPA) axis activity and anxiety-like behavior in adult offspring (9–13).

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Abbreviations: ACTH-R, ACTH receptor; BETA, betamethasone; CBG, corticosteroid-binding globulin; DEX, dexamethasone; dG, days gestation; DNase, deoxyribonuclease; GC, glucocorticoid; GR, GC receptor; HPA, hypothalamic-pituitary adrenal; 3β-HSD, 3β-hydroxysteroid dehydrogenase-isomerase; 11β-HSD2, 11β-hydroxysteroid dehydrogenase type 2; PC-1, proconvertase type 1; $P450C_{17}$, 17α-hydroxylase/17,20 lyase/17,20 desmolase; POMC, proopiomelanocortin; StAR, steroidogenic acute regulatory protein.

Similar effects of DEX and related GC have been reported in sheep, pigs, and guinea pigs (14–17). Although the timing during development of GC sensitivity varies among species, the outcomes are broadly similar.

In the ovine fetus, low-dose DEX infusion in mid to late pregnancy altered the basal set point of the HPA axis and enhanced fetal HPA axis responses to acute stress (18). We have shown previously that maternal administration of synthetic GC given to sheep late in gestation at therapeutic levels reduced prenatal (19) and postnatal weight up to 3 months of age (3). Maternal betamethasone (BETA) significantly increased basal plasma ACTH, cortisol, and cortisol-binding capacity in the fetus late in gestation (19) and increased HPA responsiveness at 1 yr of age (20). It is unknown, however, whether exposure to GC early in gestation produces similar effects on HPA axis development. This may represent a window of vulnerability. It is also unknown whether such changes in HPA activity are mediated through DEX-induced changes in pituitary output of ACTH or in adrenal steroidogenesis.

We hypothesized that synthetic GC (DEX) administration in early pregnancy would alter HPA development and function. Based on previous studies (21, 22), we considered that these effects could be sex specific. To address these issues, pregnant sheep were administered an amount of DEX analogous to that used in human subjects in the first third of pregnancy but given as a 2-d bolus, in contrast to continuous clinical treatment with GC over weeks as in cases of congenital adrenal hyperplasia. We determined effects on circulating levels of ACTH and cortisol in the fetus and measured concomitant changes in pituitary proopiomelanocortin (POMC) and proconvertase type 1 and 2 (PC-1 and -2) and GC receptor (GR). To determine effects on fetal adrenal function, we measured fetal adrenal expression of 17α-hydroxylase/17,20 lyase/17,20 desmolase ($P450C_{17}$), 3β-hydroxysteroid dehydrogenase-isomerase (3β-HSD), ACTH receptor (ACTH-R), and steroidogenic acute regulatory protein (StAR). Because DEX-induced changes in HPA function may be in part mediated through effects on hepatic corticosteroid-binding globulin (CBG) and/or placental 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2), we also measured changes in liver CBG and placenta 11β-HSD2 mRNA levels in control and treated animals.

Materials and Methods

Animals and tissues

All experimental procedures were approved by the Animal Experimentation Ethics Committee of the University of Western Australia and/or the Western Australian Department of Agriculture.

Prenatal treatments

Pregnant Merino ewes (*Ovis aries*) with singleton pregnancies (total n = 119) of known gestational age were allocated randomly to receive maternal injections of saline (control) or DEX. DEX was chosen for those studies because it is one of the most extensively studied corticosteroids used for accelerating fetal maturation and has been employed widely in experimental studies to treat central nervous system disorders and to attenuate the development of bronchopulmonary dysplasia (23–30). Maternal DEX (Mayne Pharma, Victoria, Australia) injections were given im in a dose of 0.14 mg/kg ewe weight, consisting of four im injections at 12-h intervals over 48 h on 40–41 d gestation (dG) (term 150 dG). Control animals received saline injections of a comparable volume (2 ml saline/ewe).

Tissue collection

Pregnant ewes were euthanized at 49–51 (50), 101–103 (100), 125–127 (125), and 140–142 (140) dG with a captive bolt. The fetuses were delivered by cesarean section, killed with an overdose of pentobarbitone, and weighed. Maternal (jugular) and fetal (cardiac at 50 dG or umbilical arterial) blood samples, fetal pituitaries, adrenal, liver, and placental tissues were collected. The removal of the hippocampus was done after removal of a hypothalamic block on the ventral surface of the brain. The hippocampus is clearly visible and easily dissected. This procedure has been published previously (31, 32). Pituitaries for *in situ* hybridization were slow frozen on dry ice. Adrenals, livers, and placentas were snap frozen in liquid nitrogen before storage at −80 C. Blood samples were centrifuged immediately at 4 C and plasma stored at −80 C until assayed. Other major fetal organs were removed, weighed, and collected for use in other studies.

Plasma measurements of cortisol and ACTH levels

Plasma immunoreactive ACTH concentrations were measured using a commercial RIA kit that detects $ACTH_{1\text{-}39}$ (Diasorin, Minneapolis, MN) and has been validated previously for use in sheep (33–35). Minimal detectable dose for ACTH was 2 pg/ml. The ACTH antibody cross-reacted less than 0.01% with α-MSH, β-MSH, β-endorphin, and β-lipotrophin. All samples were run in a single assay, and the intraassay coefficient of variation was 6.8%. Plasma cortisol concentrations were quantified using a commercial RIA kit (Diasorin) validated previously for use in sheep (36). The cortisol antibody cross-reacted 100% with cortisol and less than 0.04% with corticosterone, aldosterone, cortisone, and progesterone. All samples were run in a single assay, and the intraassay coefficient of variation was 3.9%.

In situ hybridization of fetal pituitary POMC, PC-1, PC-2, and GR mRNA

The method for *in situ* hybridization has been described previously in detail (19, 31, 37). Briefly, frozen pituitaries of 100, 125, and 140 dG were sectioned (12-μm coronal sections) using a cryostat (Cryostat CM 3000; Leica Microsystems, Richmond Hill, Canada) and mounted onto poly-γ-lysine-coated (Sigma Chemical Co., St. Louis, MO) slides, dried, and fixed in 4% paraformaldehyde for 5 min, rinsed in PBS (twice for 1 min), dehydrated in an alcohol series, and stored in 95% alcohol at 4 C until required for hybridization. Pituitary organ size at 50 dG did not permit *in situ* hybridization investigation at this time point. Antisense probes were generated complementary to bases

504-549 of the porcine POMC gene (33), bases 231-275 of the porcine PC-1 gene (38), bases 153-197 of the porcine PC-2 gene (39), and bases 146-191 of the ovine GR gene (40). All probes have been characterized and used previously (19, 31, 33, 41). Pituitary sections were incubated with α-^{35}S-labeled, 45-mer oligonucleotide antisense probes (POMC pars intermedia, 5 h; POMC pars distalis, 13 h; PC-1 pars intermedia, 19 d; PC-1 pars distalis, 19 d; PC-2, 10 d; and GR, 19 d). Control slides were incubated with α-^{35}S-labeled, 45-mer oligonucleotide random sequences, which did not correspond to the antisense probes. All slides were exposed together with ^{14}C standards (American Radiochemical, St. Louis, MO) to ensure analysis within the linear range of the autoradiographic film (Biomax; Kodak, Rochester, NY). The relative OD of the signal on the film of eight to 13 sections per tissue was quantified using a computerized image analysis system (MCID Imaging System version 7.0; Imaging Research, St. Catharines, Ontario, Canada). Values represent an average density over the area measured after background values were subtracted and are presented as relative mRNA levels. Control and experimental sections were processed together to allow direct comparisons between groups. Because pituitary POMC mRNA is distributed regionally, analysis of the superior region (region around the pars intermedia) and the inferior region (region at the base of the pars distalis) was performed separately in addition to analysis of the entire pars distalis.

Quantitative RT-PCR assay for ACTH-R, StAR, 3β-HSD, P450C$_{17}$, and CBG

RNA extraction and RT-PCR

Total adrenal and liver RNA was extracted using the RNeasy Midi kit (QIAGEN, Clifton Hill, Victoria, Australia). Possible genomic DNA contamination was removed from each sample using a deoxyribonuclease (DNase) treatment (Ambion, Austin, TX). Briefly, samples were incubated in 10× DNase I buffer and recombinant DNase I for 25 min at 37 C. Samples were eluted through microcolumns and then incubated with DNase inactivation reagent, centrifuged at 10,000 × *g*, and stored at −80 C. Where it was apparent that RNA was degraded (integrity gel), this sample was removed from the analysis. RNA purity was determined by spectrophotometry from the OD260/OD280 ratio, and RNA concentrations were quantified from OD260 measurements. RNA was stored at −80 C until required for use. RNA (1 μg) was reverse transcribed in a 20-μl reaction mixture containing 5× buffer (Invitrogen, Montreal, Canada), 0.1 M dithiothreitol (Invitrogen), 10 mM dNTP (Invitrogen), 0.5 μg/μl oligo(deoxythymidine)12–18 primers (Invitrogen), and 200 U/μl SuperSricpt II (Invitrogen). The RT reactions were carried out in a Peltier thermal cycler (Bio-Rad Laboratories, Inc., Hercules, CA) at 65 C for 5 min, 42 C for 2 min, 42 C for 50 min, and 70 C for 15 min. To remove RNA complementary to cDNA, ribonuclease H (Invitrogen) was used and incubated at 37 C for 20 min. A nonamplification control sample containing no RNA was reverse transcribed to provide a negative control from the RT reaction in downstream applications. For the quantification of adrenal ACTH-R, StAR, P450C$_{17}$, and 3β-HSD mRNA levels and the endogenous references 18S rRNA (18S) and β-actin, a quantitative PCR assay was performed (Rotor-Gene 3000; Corbett Research, Sydney, Australia). For quantitative PCR, all primers were either designed using Primer 3.0 (for 18S, accession no. AF176811; for StAR, accession no. NM_001009243; for 3β-HSD, accession no. NM_174343.2) or have been used previously (42, 43) on ovine tissue (ACTH-R, P450C$_{17}$, CBG, and β-actin). The following primer sequences were used: ACTH-R (forward, 5′-ACA TGG GTT ACC TCG AGC C-3′; reverse, 5′-AGA TTG TGA TGT AGC GGT CA-3′), StAR (forward, 5′-TGC GTG GAT TTA TCA GGT TC-3′; reverse, 5′-CAA GCT CTT GGT CGC TGT AG-3′), P450C$_{17}$ (forward, 5′-TGA TGA TTG GAC ACC ACC AGT TG-3′; reverse, 5′-AGA GAG AGA GGC TCG GAC AGA TC-3′), 3β-HSD (forward, 5′-TAA CAA CGG CAT CCT GAC-3′; reverse, 5′-AAG CCC CAT TCT TTG CTC-3′), CBG (forward, 5′-TGT GGG TGC CCA TGA TGT TC-3′, reverse 5′-CAG CGC AGT GAT GAC CGA GT-3′), 18S (forward, 5′-GCT ACC ACA TCC AAG GAA GG-3′; reverse, 5′-GCT CCC AAG ATC CAA CTA CG-3′), and β-actin (forward, 5′-CGG GAT CCA TCC TGC GTC TGG ACC TG-3′; reverse, 5′-GGA ATT CGG AAG GAA GGC TGG AAG AG-3′). PCR were carried out in 20-μl volumes consisting of 10× IMMUNO buffer (Bioline, Randolph, MA), 3 mM $MgCl_2$ (Bioline), 10 mM dNTPs (Invitrogen), 1.0 μM forward primer, 1.0 μM reverse primer, SYBR Green (1:2000; Fisher Biotech, West Perth, Western Australia, Australia), and 5 U/μl IMMOLASE DNA polymerase (Bioline). ACTH-R, P450C$_{17}$, and 3β-HSD cDNA were amplified using the following cycling conditions after gradient PCR optimization: 95 C for 7 min for one cycle and 95 C for 15 sec, 60 C for 15 sec, and 72 C for 15 sec for 45 cycles. StAR cDNA was amplified using the following cycling conditions after gradient PCR optimization: 95 C for 7 min for one cycle and 95 C for 8 sec, 60 C for 15 sec, and 72 C for 10 sec for 45 cycles. CBG cDNA was amplified using the following cycling conditions after gradient PCR optimization: 95 C for 7 min for one cycle and 95 C for 30 sec, 60 C for 30 sec, and 72 C for 30 sec for 40 cycles. Melting-curve analysis demonstrated a single PCR product for adrenal ACTH-R, StAR, P450C$_{17}$, 3β-HSD, CBG, β-actin, and 18S, and this was confirmed by gel electrophoresis as the presence of a single band at the appropriate molecular weight and confirmed by sequencing of the cDNA PCR products (data not shown). All samples for each gene of interest were run in duplicate in a single assay, and assays were repeated at least twice. Normalization of values to β-actin and 18S as housekeeping genes gave similar results (data not shown here). Therefore, expression levels of the genes of interest in each sample are presented relative to the internal standard and then normalized to β-actin (44). Intraassay coefficients of variation were 7.2% for ACTH-R, 5.3% for StAR, 13.6% for P450C$_{17}$, 8.6% for 3β-HSD, 4.0% for CBG, 8.4% for β-actin, and 12.7% for 18S. β-Actin and 18S were used to calculate relative gene mRNA levels.

Quantitative RT-PCR assay for 11β-HSD2

At each gestational age, a subset of placental tissue was used for measurement of 11β-HSD2 mRNA using protocols reported previously (45). The quantitative real-time PCR mix was 1 μl cDNA, 0.5 μM of each paired primer, 12.5 μl Platinum quantitative PCR SuperMix-UDG (Invitrogen, Burlington, Ontario, Canada), 0.8 μl SYBR Green (1:1000 diluted; Invitrogen), and 9.7 μl diethylpyrocarbonate-H_2O in 25 μl final volume. The 11β-HSD2 primer (NM_001009460) has been used previously (45). Samples were assayed in duplicate in each of three separate runs. For each duplicate, 22 μl were used from the prepared 25 μl to ensure equal volume loading. Relative levels of 11β-HSD2 were normalized to the housekeeping gene β-actin. For negative control, the target cDNA template was replaced with water in the complete reaction mix. A nonamplification control was used in which water replaced RNA during cDNA synthesis. The PCR

TABLE 1. Effects of early maternal DEX on fetal anthropometric data

	50 dG		100 dG		125 dG		140 dG	
	Control (6 F, 10 M)	**DEX (6 F, 10 M)**	**Control (11F, 5 M)**	**DEX (5 F, 8 M)**	**Control (9 F, 6 M)**	**DEX (4 F, 7 M)**	**Control (7 F, 7 M)**	**DEX (4 F, 6 M)**
Fetus								
F	13.68 ± 0.95	14.25 ± 0.55	**888.00 ± 23.37**[a]	**853.20 ± 52.28**[a]	2787.44 ± 116.84	2694.25 ± 143.58	4681.27 ± 121.97	4511.25 ± 198.00
M	14.57 ± 0.70	15.01 ± 0.46	975.60 ± 47.07	936.43 ± 61.05	2988.33 ± 86.10	2876.50 ± 178.48	5168.00 ± 135.64	4847.68 ± 198.63
Gender	Ø	Ø	**M-C > F-C**[a]	**M-D > F-D**[a]	Ø	Ø	**M-C > F-C**[a]	Ø
Hippocampus								
F	Not measured	Not measured	$6.79^{-4} \pm 2.69^{-5}$	$7.40^{-4} \pm 2.40^{-5}$	$3.50^{-4} \pm 1.32^{-5}$	$3.69^{-4} \pm 8.45^{-6}$	$2.54^{-4} \pm 3.71^{-5}$	$2.72^{-4} \pm 1.07^{-5}$
M			$5.78^{-4} \pm 8.63^{-5}$	$6.91^{-4} \pm 5.18^{-5}$	$3.32^{-4} \pm 1.62^{-5}$	$3.65^{-4} \pm 1.12^{-5}$	**$2.40^{-4} \pm 1.01^{-5}$**[a]	**$2.85^{-4} \pm 1.45^{-5}$**[a]
Gender			**F-C > M-C**[a]	Ø	Ø	Ø	Ø	Ø
Pituitary								
F	$2.12^{-4} \pm 4.71^{-5}$	$2.48^{-4} \pm 5.58^{-5}$	$6.24^{-4} \pm 2.45^{-6}$	$6.17^{-4} \pm 1.40^{-6}$	$3.66^{-5} \pm 2.21^{-6}$	$4.05^{-5} \pm 2.60^{-6}$	$3.38^{-5} \pm 1.51^{-6}$	$3.20^{-5} \pm 2.20^{-6}$
M	$1.67^{-4} \pm 2.48^{-5}$	$2.38^{-4} \pm 4.41^{-4}$	**$5.14^{-4} \pm 3.46^{-2}$**[a]	**$6.07^{-4} \pm 2.86^{-6}$**[a]	$3.41^{-5} \pm 1.84^{-6}$	$4.43^{-5} \pm 5.50^{-6}$	$2.83^{-5} \pm 2.63^{-6}$	$3.15^{-5} \pm 1.37^{-6}$
Gender	Ø	Ø	**F-C > M-C**[a]	Ø	Ø	Ø	Ø	Ø
LT ADR								
F	$6.13^{-3} \pm 4.29^{-5}$	$5.01^{-3} \pm 2.69^{-5}$	$9.60^{-5} \pm 6.69^{-6}$	$9.22^{-5} \pm 1.27^{-5}$	$6.14^{-5} \pm 4.99^{-6}$	$5.81^{-5} \pm 1.02^{-6}$	$5.28^{-5} \pm 2.93^{-6}$	$4.52^{-5} \pm 1.88^{-6}$
M	$6.05^{-3} \pm 2.56^{-5}$	$5.70^{-3} \pm 6.40^{-5}$	$9.18^{-5} \pm 4.90^{-6}$	$8.01^{-5} \pm 6.30^{-6}$	$4.64^{-5} \pm 3.76^{-6}$	$4.95^{-5} \pm 5.30^{-6}$	$4.13^{-5} \pm 3.27^{-6}$	$3.61^{-5} \pm 3.29^{-6}$
Gender	Ø	Ø	Ø	Ø	**F-C > M-C**[a]	Ø	**F-C > M-C**[a]	Ø
RT ADR								
F	$6.81^{-4} \pm 5.90^{-5}$	$7.17^{-4} \pm 6.82^{-5}$	$8.75^{-5} \pm 7.94^{-6}$	$1.01^{-4} \pm 2.47^{-6}$	**$6.80^{-5} \pm 5.76^{-6}$**[a]	**$4.97^{-4} \pm 4.0^{-6}$**[a]	$5.11^{-5} \pm 4.52^{-6}$	$4.33^{-5} \pm 2.46^{-6}$
M	$6.47^{-4} \pm 3.27^{-5}$	$6.12^{-4} \pm 5.55^{-4}$	$8.36^{-5} \pm 4.32^{-6}$	$8.60^{-5} \pm 6.12^{-6}$	$4.34^{-5} \pm 3.10^{-6}$	$4.60^{-5} \pm 5.70^{-6}$	$3.40^{-5} \pm 2.98^{-6}$	$3.58^{-5} \pm 2.30^{-6}$
Gender	Ø	Ø	Ø	Ø	**F-C > M-C**[a]	Ø	**F-C > M-C**[a]	Ø
Liver								
F	$7.18^{-2} \pm 3.60^{-3}$	$6.46^{-2} \pm 3.13^{-3}$	$4.67^{-2} \pm 8.60^{-4}$	$4.81^{-2} \pm 1.07^{-3}$	$3.02^{-2} \pm 3.62^{-3}$	$2.89^{-2} \pm 2.59^{-3}$	$2.59^{-2} \pm 1.84^{-3}$	$2.36^{-2} \pm 1.39^{-3}$
M	$6.27^{-2} \pm 6.45^{-3}$	$6.60^{-2} \pm 1.71^{-3}$	$4.61^{-2} \pm 1.81^{-3}$	$4.86^{-2} \pm 2.28^{-3}$	$2.94^{-2} \pm 1.12^{-3}$	$3.28^{-2} \pm 1.49^{-3}$	$2.61^{-2} \pm 0.10^{-5}$	$2.53^{-2} \pm 1.31^{-3}$
Gender	Ø	Ø	Ø	Ø	Ø	Ø	Ø	Ø
Placenta								
F	59.1 ± 9.9	40.3 ± 10.3	472.9 ± 35.8	476.7 ± 40.7	414.9 ± 36.2	431.5 ± 58.7	499.15 ± 68.1	512.9 ± 29.0
M	66.1 ± 6.8	52.8 ± 8.9	501.2 ± 55.3	395.7 ± 26.1	404.1 ± 33.6	465.7 ± 42.3	575.9 ± 45.9	570.7 ± 62.9
Gender	Ø	Ø	Ø	Ø	Ø	Ø	Ø	Ø

Organ weights are presented in grams, normalized to fetal weight. Females (F), males (M), and gender differences (gender) are represented as mean ± SE. Data were analyzed by univariate ANOVA followed by two-way ANOVA. Significant differences between control (C) and treatment (D) and gender differences are represented in *bold*. Total n = 111 [61 (31 F, 30 M) controls and 50 DEX (22 F, 28 M)]. LT ADR, Left adrenal weight; RT ADR, right adrenal weight; Ø, no significant gender differences.

[a] $P < 0.05$.

protocol included initial denaturation of 5 min at 95 C followed by 45 cycles of denaturation for 20 sec at 95 C, annealing for 20 sec at 56 C, and extension for 20 sec at 72 C. Results are expressed as relative mRNA levels.

Statistical analysis

Statistical analysis was performed using SigmaStat Statistical Software (SigmaStat version 2.03; SYSTAT Software Inc., Chicago, IL) and SPSS version 14.0.1 (SPSS Inc., Chicago, IL). Data were analyzed first for normality and equal variance (Levene's test). Data that were not normally distributed were log transformed to achieve normality. Data sets were analyzed using a full factorial general linear model (univariate ANOVA) with gender, treatment, and age as fixed factors. A two-way ANOVA followed by a *post hoc* Tukey test was used to determine time and treatment effects on plasma ACTH and cortisol, RT-PCR analysis of adrenal tissue, and *in situ* hybridization analysis of fetal pituitary tissue. Data are presented as mean ± SE. Statistical significance was accepted for $P < 0.05$.

Results

Effect of prenatal DEX exposure on fetal weight and organ weights

In all cases, males were bigger than females (100 and 140 dG, $P < 0.05$; Table 1). In controls, females at 100 dG were longer (crown rump length) and had larger abdominal circumference at 140 dG than males ($P < 0.05$). DEX-exposed fetuses had reduced fetal weights at 100 dG ($P < 0.05$; not shown here) and increased total brain weight adjusted to fetal weight at 100 dG compared with control ($P < 0.05$). DEX-treated males had higher pituitary weights (at 100 dG) and higher hippocampus weights adjusted to fetal weights compared with controls at 140 dG ($P < 0.05$). Total adrenal weight in females was reduced in DEX fetuses at 125 and 140 dG compared with controls ($P < 0.05$). Control females had larger adrenals than males at 125 and 140 dG ($P < 0.05$), but this sex-specific effect was not observed in DEX-treated animals. Heart, liver, pancreas, kidney, and total perirenal fat weights were similar between groups (data not shown here). No significant differences in placental weights were found.

Effects of prenatal DEX exposure on concentrations of fetal plasma ACTH and cortisol

There was a significant effect of sex ($P = 0.001$) and age ($P < 0.001$) and a sex-by-age interaction ($P = 0.001$) on fetal plasma ACTH levels (Fig. 1A); in control female fetuses, fetal plasma ACTH concentrations increased across gestation (50 *vs.* 125 dG and 125 *vs.* 140 dG, $P = 0.029$). ACTH concentrations did not differ between treatment groups for either sex (Fig. 1A). In all fetuses, plasma cortisol concentrations were highest at 50 dG and then significantly decreased on 100 dG ($P < 0.05$) before increasing again at 140 dG ($P < 0.05$; Fig. 1B). DEX exposure resulted in significantly lower plasma cortisol levels at 50 dG in fetal males ($P < 0.05$) but not at 100 and 125 dG. At 140 dG, plasma cortisol levels were significantly elevated in both male and female DEX-treated fetuses compared with controls, this effect was more pronounced in females ($P = 0.002$; Fig. 1B).

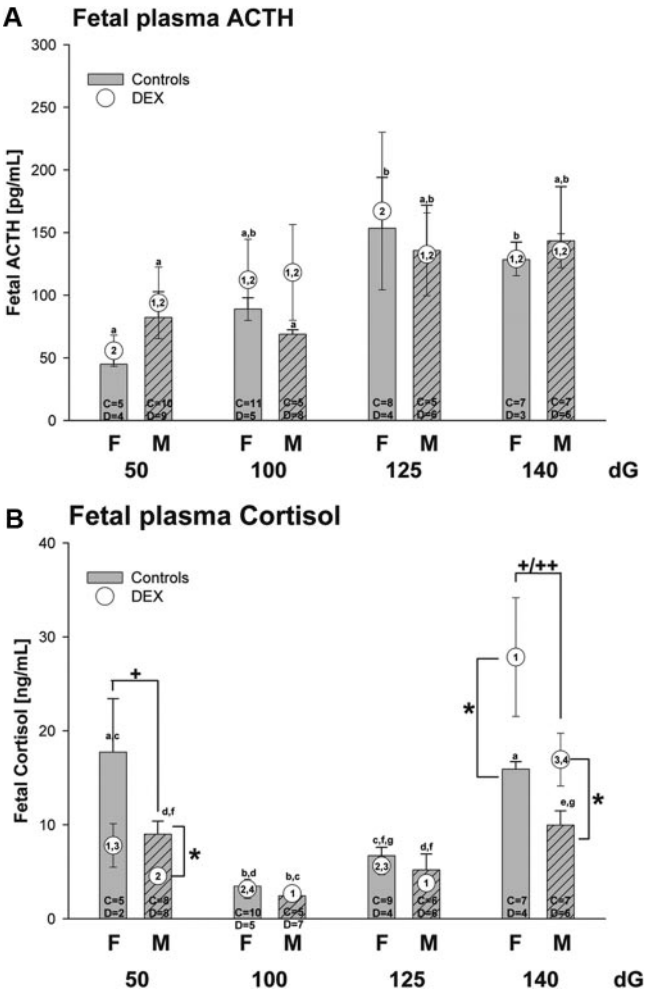

FIG. 1. Fetal umbilical cord plasma ACTH (A) and cortisol (B) level. Controls (C) are represented by *vertical bar charts* [*gray*, females (F); *gray striped*, males (M)] and DEX-treated (D) animals as *white circles*. *Different letters* (a–g) on the histogram represent significant differences in control groups across gestation. Significant differences across gestation are represented in *numbers* (1, F control; 2, F DEX; 3, M control; 4, M DEX; $P < 0.05$). Significant differences between control and treatment are represented as indicated: *, $P < 0.05$. Gender differences are also indicated: +, control; ++, DEX; $P < 0.05$.

Effect of prenatal DEX exposure on levels of pituitary POMC, PC-1, PC-2, and GR mRNA

Pars distalis

In all fetuses, POMC and PC-1 mRNA were expressed in pars distalis and pars intermedia; GR mRNA was expressed only in pars distalis and PC-2 mRNA only in pars intermedia as described before (37). Early DEX treatment had little effect on mRNA expression of these genes in pars

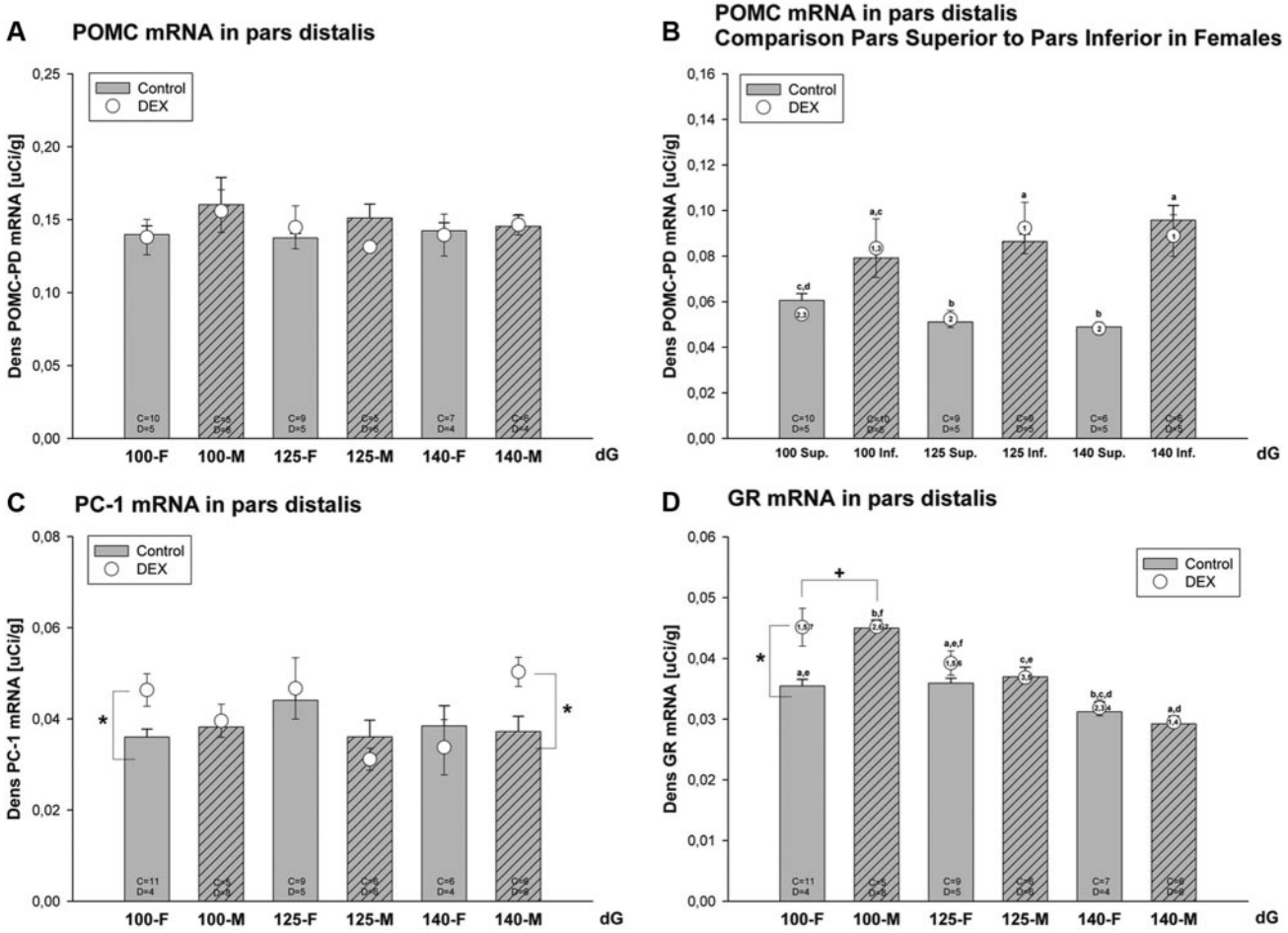

FIG. 2. Pituitary mRNA expression in pars distalis. Panel A, POMC; panel B, POMC pars superior *vs.* pars inferior; panel C, PC-1; panel D, GR. Controls are represented by *vertical bar charts* [*gray*, females (F); *gray striped*, males (M)] and DEX-treated animals as *white circles*. Significant differences between control (C) and treatment (D) are indicated: *, $P < 0.05$. Gender differences are also indicated: +, control; $P < 0.05$. *Letters* in panels B and D represent differences in mRNA expression in controls, and *numbers* represent differences in DEX-treated animals.

distalis. POMC mRNA expression in the pars distalis was differentially expressed such that levels were significantly higher in the inferior region compared with the superior region ($P < 0.001$; Fig. 2B). There was no effect of early DEX treatment on POMC mRNA expression in males or females compared with controls (Fig. 2A) or on the distribution of POMC mRNA. PC-1 mRNA levels in the pars distalis were relatively unchanged between 100 and 140 dG (Fig. 2C). DEX-treated females had significantly higher PC-1 mRNA levels at 100 dG compared with controls ($P = 0.012$), and DEX-treated males had significantly higher levels of PC-1 mRNA at term ($P = 0.009$; Fig. 2C). Control males had significantly higher levels of GR mRNA at 100 dG compared with females ($P < 0.001$; Fig. 2D), but DEX significantly increased GR mRNA in females compared with controls at 100 dG ($P < 0.001$; Fig. 2D).

Pars intermedia

POMC mRNA level in males and females followed previously reported patterns (37). DEX significantly decreased POMC mRNA level at 100 dG in both sexes and at 125 dG in males compared with controls ($P < 0.02$; Fig. 3A). PC-1 mRNA levels significantly increased between 100 and 140 dG in both controls and DEX-treated fetuses ($P < 0.05$; Fig. 3B). PC-2 mRNA levels significantly increased between 100 and 140 dG in both female (control and DEX, $P < 0.05$) and male (DEX, $P < 0.05$; Fig. 3C) fetuses, but there were no effects of sex, age, or DEX treatment.

Effect of prenatal DEX exposure on relative levels of adrenal ACTH-R, StAR, P450C$_{17}$, and 3β-HSD mRNA

In females and males, ACTH-R, StAR, and 3β-HSD relative mRNA levels significantly increased in controls between 50 and 140 dG ($P < 0.05$; Fig. 4, A, B, and D). At 125 dG, males had significantly higher ACTH-R mRNA levels compared with females ($P = 0.004$; Fig. 4A), but this was abolished after early DEX treatment (Fig. 4A; $P < 0.01$). No sex effect at different ages in controls was found for either 3β-HSD or StAR relative mRNA levels. In both

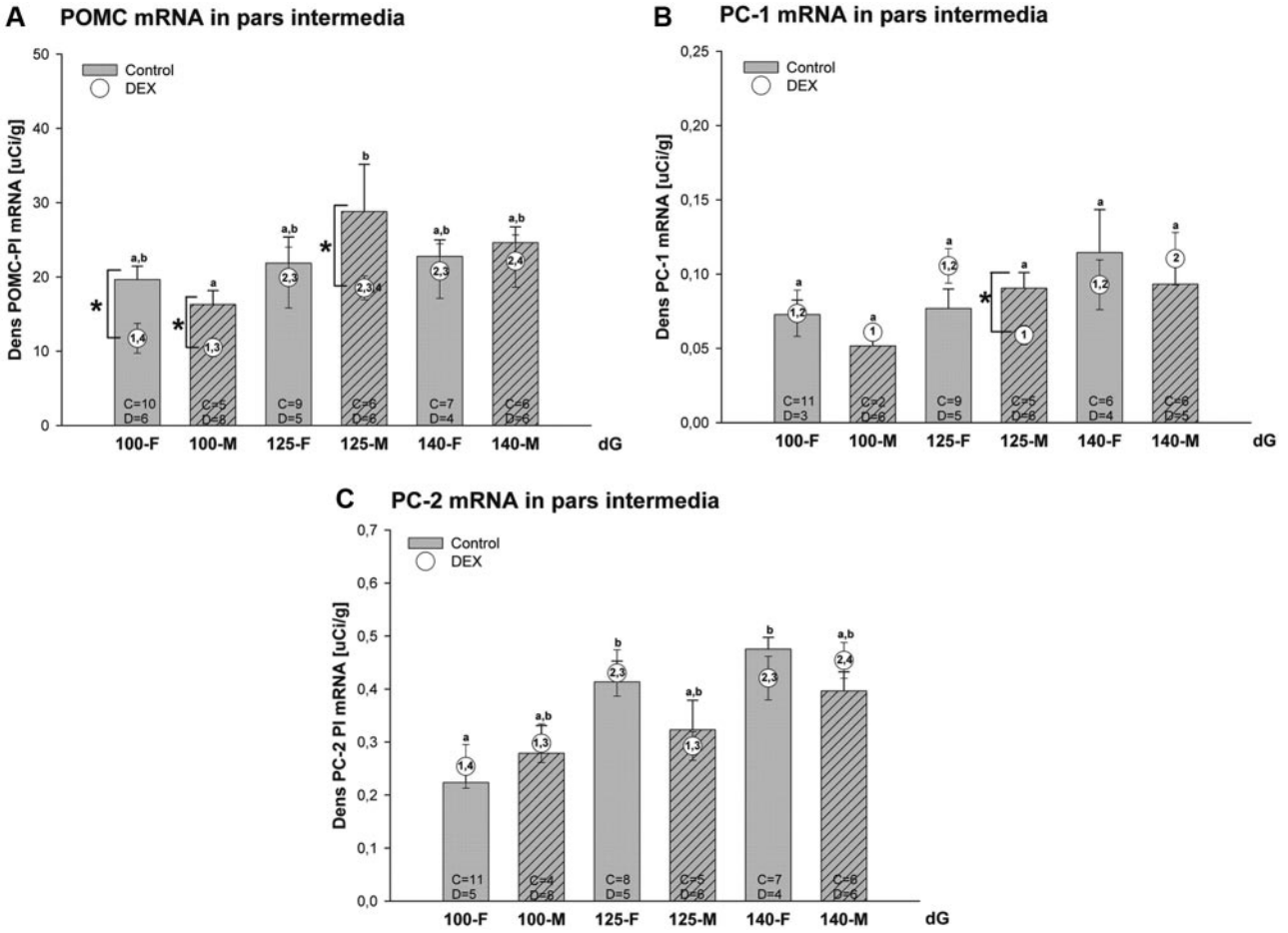

FIG. 3. Pituitary mRNA expression in pars intermedia. Panel A, POMC; panel B, PC-1; panel C, PC-2. Controls are represented by *vertical bar charts* [*gray*, females (F); *gray striped*, males (M)] and DEX-treated animals as *white circles*. Significant differences between control (C) and treatment (D) are indicated: *, $P < 0.05$. *Letters* represent differences in mRNA expression in controls, and *numbers* represent differences in DEX-treated animals.

sexes $P450C_{17}$ mRNA levels were higher at 50 dG than at 100 dG but increased again at 125 and 140 dG ($P < 0.05$; Fig. 4C). DEX suppressed $P450C_{17}$ in females at 50 dG ($P = 0.027$), but at 140 dG, $P450C_{17}$ was significantly higher in females after early DEX treatment compared with controls ($P = 0.003$; Fig. 4C). No effect of DEX on $P450C_{17}$ mRNA levels was found in males. 3β-HSD mRNA was also significantly higher at 140 dG in females fetuses after early maternal DEX treatment ($P = 0.025$; Fig. 4D), and DEX-treated females had significantly higher 3β-HSD mRNA levels compared with treated males ($P < 0.05$; Fig. 4D).

Effect of prenatal DEX exposure on relative mRNA levels of hepatic CBG

Relative levels of hepatic CBG mRNA decreased significantly between 50 and 100 dG in both female and male fetuses and then significantly increased to 140 dG ($P < 0.05$; Fig. 5). DEX treatment decreased relative CBG mRNA levels in males compared with controls at 50 dG ($P < 0.05$; Fig. 5). There was no effect of DEX on CBG mRNA levels in females or in males at other gestational ages.

Effect of prenatal DEX exposure on relative mRNA levels of placental 11β-HSD2

In control males, relative placental 11β-HSD2 mRNA levels significantly increased between 50 and 100 and 125 and 140 dG ($P = 0.001$), and in DEX-treated males, levels increased between 50 and 100 and 140 dG ($P < 0.05$). In male fetuses, DEX exposure significantly increased placental 11β-HSD2 mRNA levels compared with controls at 50 dG ($P = 0.017$) and at 140 dG ($P = 0.047$). In contrast, in females, placental 11β-HSD2 mRNA levels did not change with advancing gestation and levels were unaffected by DEX exposure.

Discussion

We have presented novel information demonstrating that DEX exposure early in gestation alters the developmental

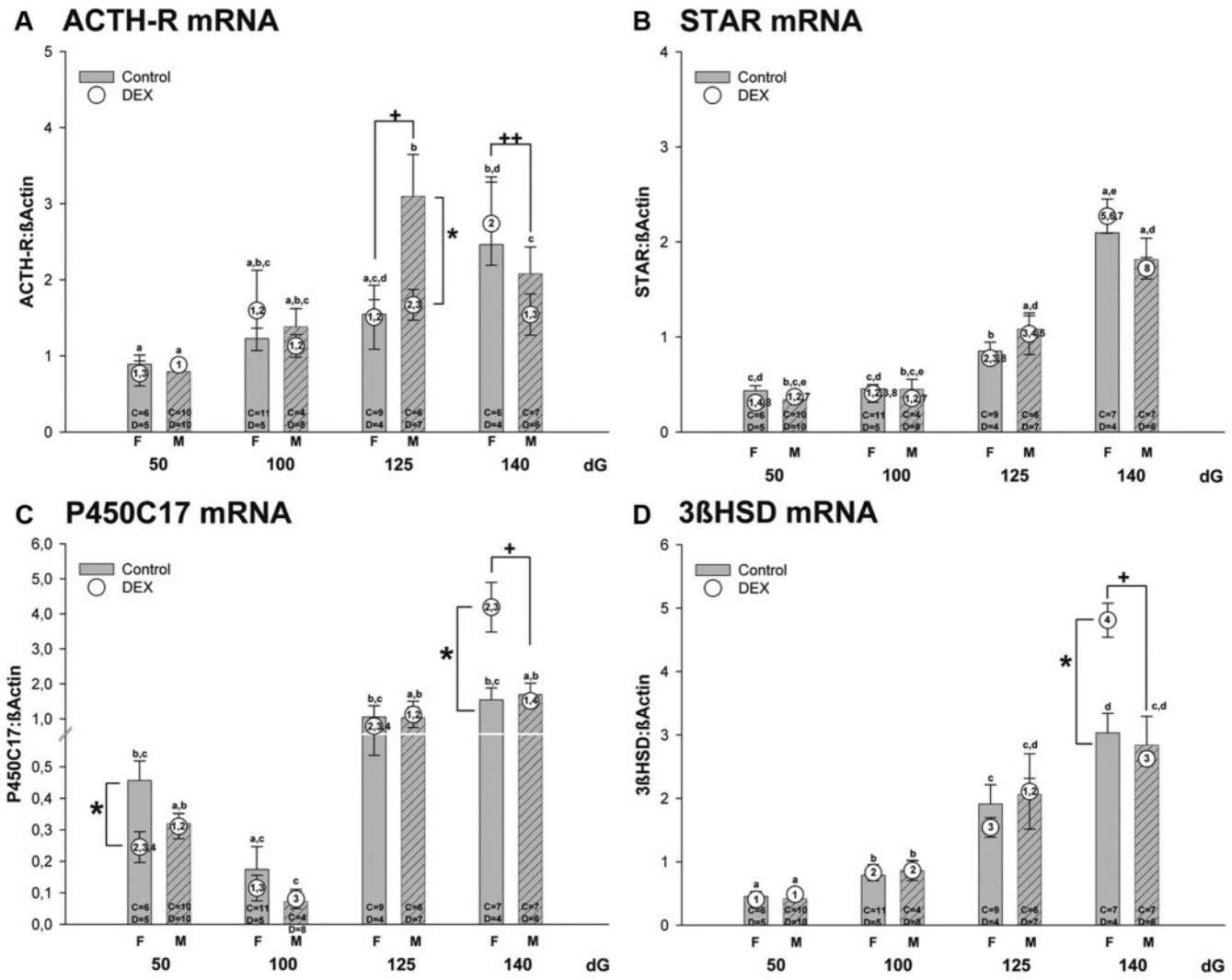

FIG. 4. Adrenal mRNA expression relative to β-actin. Panel A, ACTH-R; panel B, StAR; panel C, P450C_{17}; panel D, 3β-HSD. Controls are represented by *vertical bar charts* [*gray*, females (F); *gray striped*, males (M)] and DEX-treated animals as *white circles*. Significant differences between control (C) and treatment (D) are indicated: *, $P < 0.05$. Gender differences are also indicated: +, control; ++, DEX; $P < 0.05$. *Letters* represent differences in mRNA expression in controls, and *numbers* represent differences in DEX-treated animals across gestation. 3β-HSD mRNA significantly increased across gestation in controls and DEX-treated animals.

trajectory of the fetal sheep HPA axis culminating in an increased activation of key steroidogenic enzymes in the fetal adrenal by term. In females, it appears that the DEX-induced increase in circulating plasma cortisol concentrations may be attributed, in part, to increased levels of adrenal steroidogenic expression. Additionally, we have demonstrated that DEX exposure results in a sex-dependant increase in placental 11β-HSD2 mRNA levels as early as 50 dG.

We have shown that DEX treatment early in pregnancy initially suppressed cortisol output, which is not dissimilar to other reported observations in this species (46). Clinically, antenatal GC administration is known to suppress fetal HPA activity, resulting in lower fetal cortisol levels, hypothesized to be secondary to central negative feedback. However, there was no change in fetal ACTH at 50 dG after maternal DEX, perhaps implying a direct action of early GC administration in the fetal adrenal gland.

Fetal adrenal differentiation and growth is dynamic, with a rapid growth period between 40 and 60 dG, followed by relative adrenal quiescence between 90 and 120 dG and an unresponsiveness to ACTH (47–49). The fetal adrenal growth spurt then continues from 120 dG until term (49–51). In the present study, ACTH-R, StAR, and 3β-HSD and P450C_{17} mRNA significantly increased in all fetuses, with highest levels at 140 dG ($P < 0.05$), consistent with previous studies (47, 49). We now show that elevated cortisol levels in female fetuses at term after earlier maternal DEX administration was associated with further increases in fetal adrenal P450C_{17} and 3β-HSD mRNA levels. The DEX-induced increase in cortisol concentrations in male fetuses at term was independent of the

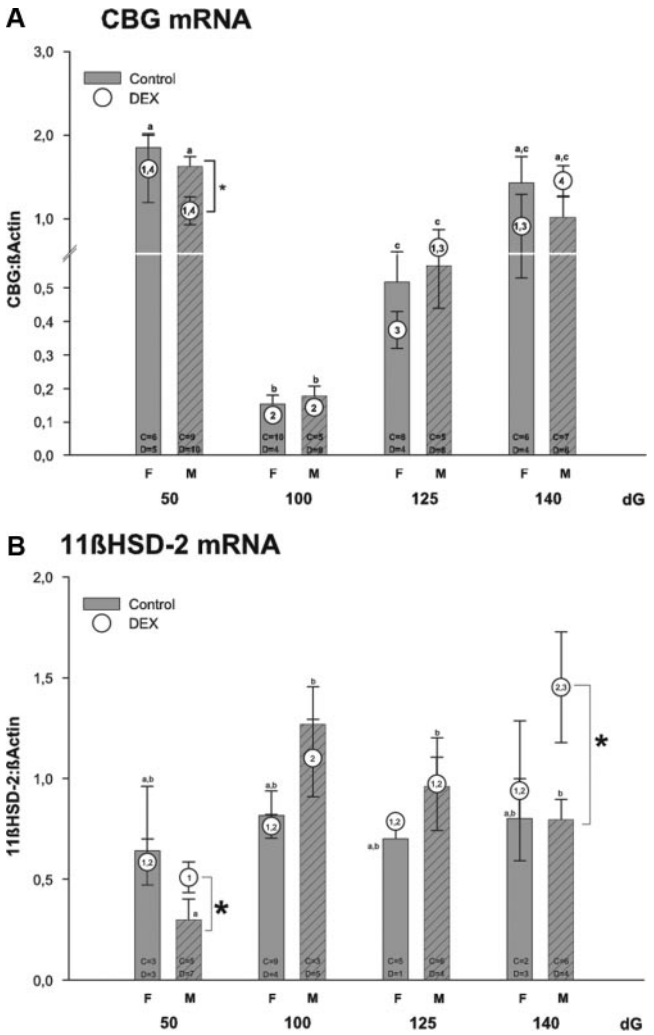

FIG. 5. Liver CBG mRNA expression (A) and placenta 11β-HSD2 mRNA expression (B) relative to β-actin. Controls are represented by *vertical bar charts* [*gray*, females (F); *gray striped*, males (M)] and DEX-treated animals as *white circles*. Significant differences between control (C) and treatment (D) are indicated: *, $P < 0.05$. Gender differences are also indicated: +, control; ++, DEX; $P < 0.05$. *Letters* represent differences in mRNA expression in controls, and *numbers* represent differences in DEX-treated animals across gestation.

enzymes that we have measured, although we did not measure enzymatic activity in the present study. It is possible that other factors present in the adrenal may affect cortisol output from these fetuses.

Sex-specific regulation of the fetal (52) and adult (43) HPA axis *per se* has been noted previously (for review, see Ref. 53), and it has been suggested that fetal sex is an important determinant of outcome after antenatal GC administration (54). Studies have suggested that female fetuses respond to early life events (such as antenatal GC or maternal asthma) in a manner that facilitates improved survival (55). Because the plasma cortisol levels at 50 dG in control females was already significantly higher than in males, one might speculate that females are more responsive to exogenous GC stimulation at this developmental time window, with downstream effects on other organ systems.

The regulation of fetal pituitary-adrenal function and downstream cortisol output is multifactorial. Centrally, hypothalamic CRH and arginine vasopressin (AVP) are the primary stimulators of corticotroph cells within the pars distalis of the pituitary that synthesize POMC and secrete ACTH (56, 57). We did not observe changes in ACTH levels in DEX-exposed fetuses consistent with a lack of change in POMC distribution or relative mRNA levels. In the pars intermedia, however, relative POMC mRNA levels were significantly reduced at 100 dG, a response that persisted in males until 125 dG. Recent evidence has suggested that urocortin 2 in the pars intermedia is regulated negatively by GC (58). It is possible that DEX-induced changes in local urocortin 2 may have influenced pars intermedia POMC levels, although this was beyond the scope of the present study. DEX administration did not result in consistent changes in either PC-1 or PC-2 levels in the pars intermedia. The variable effects of PC-1 mRNA in the pars distalis suggest that it is unlikely that our results reflect consistent changes in POMC processing, although this requires further study.

GR mRNA levels in the pars distalis significantly decreased between 100 and 140dG in males and female fetuses consistent with altered negative feedback (58, 59). Although *in vitro* experiments showed that GR expression was directly down regulated by GC (59), in the present study, maternal DEX administration was associated with increased GR mRNA levels at 100dG in the pars distalis in female fetuses. Maternal BETA late in gestation did not change GR mRNA expression in the pituitary at 125dG (35), suggesting, that GR in the sheep pituitary is regulated by GC in a more complex and dynamic mechanism than simple exposure to cortisol (32).

Our results suggest that the developmental patterns of adrenal growth and cortisol output in the fetal sheep are sex specific. Previous studies have suggested a neuroendocrine sexual dimorphism (60, 61). In rats, females have higher basal levels of plasma corticosterone than males and higher rates of adrenal corticosteroidogenesis (62). ACTH or stress produced higher and more prolonged elevated plasma corticosterone levels in females (62). In humans, males may be more vulnerable to intrauterine events, presenting with higher rates of morbidity and mortality. For example, in male fetuses, there is a greater incidence of respiratory distress syndrome, suggestive of poorer GC responsiveness in male fetal lungs compared with that of female fetuses (63).

Sex-related differences in human placental activity of 11β-HSD2 have also been reported. 11β-HSD2 activity is higher in female trophoblastic tissue than in males, sug-

gesting an increased exposure of the male fetus to GCs of maternal origin (64). Intriguingly, in the present study, we found that maternal DEX administration in early pregnancy increased placental 11β-HSD2 mRNA levels in male fetuses. This difference was present as early as 50 dG and at term. It is possible that sex-specific changes in placental 11β-HSD2 alter the relative passage of endogenous maternal cortisol to the fetus and influence its effects on fetal adrenal enzyme expression. Recently, we have found that periconceptional undernutrition in sheep was associated with reduced 11β-HSD2 in the placenta and increased HPA axis function at term (45), a problem consistent with the present results.

Repeated maternal GC injections late in gestation reduced fetal weight in sheep (6, 19, 65, 66), although evidence for such an effect in humans is conflicting at this time (67–69). Maternal DEX exposure early in gestation resulted in fetal and organ weight changes, some of which persisted to term. DEX-treated females were lighter than controls at 100 dG. DEX-treated animals had a significantly higher ponderal index at 100 and 140 dG compared with controls. Rhesus monkeys, which received BETA for 13 d late in gestation, showed increased fetal pituitary weights, reduced adrenal weights, and decreased basal cortisol concentrations (70). In our study, no differences in pituitary weights were observed immediately after DEX treatment at 50 dG, but significantly higher pituitary weights were found in DEX-treated animals at 100 dG ($P < 0.05$) compared with controls, as reported before (21). Thus, DEX treatment even for short periods in early gestation can influence trajectories of fetal development.

We have demonstrated that maternal GC administration early in pregnancy affects fetal pituitary-adrenal axis development, particularly targeting adrenal steroidogenic enzymes in females and placental 11β-HSD2 in males. Others have shown that early gestation exposure of the fetus to GC leads to programming of blood pressure, an effect that persists well into adulthood (71). It is possible that this response is attributable, at least in part, to the changes we report in HPA axis activity.

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2.1.3 Glukokortikoidexposition – Rolle der Plazenta

T. Braun, W. Meng, H. Shang, S. Li, D.M. Sloboda, L. Ehrlich, K. Lange, H. Xu, W. Henrich, J. W. Dudenhausen, A. Plagemann, J. P. Newnham and J.R.G. Challis: „ Early dexamethasone treatment induces placental apoptosis in sheep" Reproductive Sciences 2015; 22(1): 47-59[324]

Hintergrund: Sowohl die Behandlung mit GC in der Spät- als auch in der Frühschwangerschaft beim Schaf führt zu einer Beeinträchtigung der fetalen Entwicklung, Wachstumsrestriktion mit postnatalen Langzeitfolgen. Bislang konnte jedoch nicht erörtert werden, ob die zahlenmäßige Reduktion der BNC nach maternaler GC-Exposition in der Spätschwangerschaft durch eine Verminderung der BNC-Formation, einer vermehrten Migration und/oder durch Apoptose hervorgerufen wird. **Arbeitshypothese:** Die frühe maternale DEX-Therapie beeinflusst die plazentare Entwicklung und Funktion und könnte so zu den Langzeit-Veränderungen in HPA-Achse beitragen. Ein DEX induziertes Ungleichgewicht von plazentaren pro- und anti-apoptotischen Faktoren könnte hierbei eine Rolle spielen. **Ziel:** Untersuchung der Auswirkungen früher maternale DEX-Therapie auf die Anzahl, Verteilung und Funktion von BNC und die Rolle der Apoptose in der Schafsplazenta. **Methode:** Trächtige Mutterschafe mit Einlingsschwangerschaften wurden randomisiert und mit Injektionen von Kochsalzlösung (2ml, n=61) oder DEX (4x0,14mg/kg Mutterschafgewicht) am Tag 40 bis 41 der Schwangerschaft (dG) behandelt. An den Tagen 50, 100, 125 und 140 dG wurden fetales Plasma und Gewebe gesammelt. **Ergebnisse:** Bei weiblichen Feten führte die frühe maternale DEX-Behandlung zu einer signifikanten Reduktion des Fetalgewichts, die mit einer Reduktion in der Anzahl der BNC, einer Reduktion von anti-apoptotischen Markern (PCNA-mRNA) und einer Zunahme von pro-apoptotischen Markern (Bax-, p53-mRNA) am Tag 100 assoziiert war. Am Tag 125 normalisierten sich sowohl das Fetalgewicht als auch die plazentaren Parameter (Anzahl der BNC, Apoptosemarker). Des Weiteren zeigten sich nach DEX-Therapie unterschiedliche Glykosylierungsraten von oPL. Bei den männlichen Feten fanden sich zwar signifikant reduzierte oPL-Proteinlevel an den Tagen 100 und 140, diese waren jedoch unabhängig von der Anzahl der BNC oder Apoptosemarkern. Interessanterweise zeigten sich bei den fetalen und maternalen oPL-Plasmaspiegeln keine Veränderungen nach früher DEX-Therapie. **Diskussion/Schlussfolgerung:** Die geschlechtsspezifische Reduktion in der Gesamt-Zellzahl der BNC nach DEX-Therapie war bei weiblichen Feten von einer Induktion von plazentaren Apoptose-Markern begleitet (↑ Caspase-3-, ↓ PCNA-mRNA-Level). Die bei den männlichen Feten gefundenen Veränderungen in der Plazenta waren hierbei unabhängig von den untersuchten plazentaren Apoptose-Markern. Diese Daten lassen vermuten, dass eine differenzielle oPL-Abgabe der BNC an die maternale (Wanderung und Exocytose) und fetale (Sekretion) Zirkulation vorliegt, und diese durch die DEX-Therapie in unterschiedlicher Weise beeinflusst wird.

Weitere eigene, relevante Publikationen zu diesem Thema:

T. Braun, JP Newnham, JRG Challis, DM Sloboda: "Early life glucocorticoid exposure: The HPA axis, placental function and long term disease risk." Endocrine Reviews August 22, 2013 er.2013-1012[15]

J.R.G. Challis, D. M. Sloboda, S. Li, **T. Braun**, F. Bloomfield, G. Begum, A. White, F. Petraglia, J.P. Newnham: "The Role of the Placenta in Fetal Programming." Buchbeitrag in „Hormones, Intrauterine Health and Programming Series: Research and Perspectives in Endocrine Interactions, Vol. 12, Seckl, Jonathan R.; Christen, Yves (Eds.); Springer Verlag Berlin 2014, pp. 57-70[325]

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Early Dexamethasone Treatment Induces Placental Apoptosis in Sheep

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Abstract

Glucocorticoid treatment given in late pregnancy in sheep resulted in altered placental development and function. An imbalance of placental survival and apoptotic factors resulting in an increased rate of apoptosis may be involved. We have now investigated the effects of dexamethasone (DEX) in early pregnancy on binucleate cells (BNCs), placental apoptosis, and fetal sex as a determinant of these responses. Pregnant ewes carrying singleton fetuses (n = 105) were randomized to control (n = 56, 2 mL saline/ewe) or DEX treatment (n = 49, intramuscular injections of 0.14 mg/kg ewe weight per 12 hours over 48 hours) at 40 to 41 days of gestation (dG). Placentomes were collected at 50, 100, 125, and 140 dG. At 100 dG, DEX in *females* reduced BNC numbers, placental antiapoptotic (*proliferating cell nuclear antigen*), and increased proapoptotic factors (*Bax*, *p53*), associated with a temporarily decrease in fetal growth. At 125 dG, BNC numbers and apoptotic markers were restored to normal. In *males*, ovine placental lactogen-protein levels after DEX were increased at 50 dG, but at 100 and 140 dG significantly decreased compared to controls. In contrast to females, these changes were independent of altered BNC numbers or apoptotic markers. Early DEX was associated with sex-specific, transient alterations in BNC numbers, which may contribute to changes in placental and fetal development. Furthermore, in females, altered placental apoptosis markers may be involved.

Keywords

binucleate cell, placental lactogen, apoptopic markers, glucocorticoid, placenta

Introduction

The administration of synthetic glucocorticoids (GCs) is an important clinical tool used both in late pregnancy, for the management of women at risk of early preterm birth,[1-3] and in early pregnancy, in suspected cases of congenital adrenal hyperplasia (CAH) to prevent fetal virilization.[4,5] The lifelong consequences of this treatment are not fully understood.[6-10] Human fetuses with CAH, who had received dexamethasone (DEX), had normal pre- and postnatal growth[11] but showed more shyness, greater emotionality, and less sociability than unexposed children.[12]

Previous studies in sheep have shown that maternal *intravenously* DEX treatment between 26 and 28 days of gestation (dG) did not result in fetal organ weight changes at 130 dG[13] but were associated with enhanced coronary artery vascular reactivity with 4 months of age,[14] altered brain renin–angiotensin function[15] and hyperinsulinemia in response to glucose challenge at the age of 4 years.[16] Female offspring demonstrated hypertension, which was still evident at the age of 7 years.[17] Maternal *intramuscular* (im) DEX treatment between

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40 and 41 dG in pregnant sheep resulted in changes in fetal and organ weights, some of which persisted into later life.[18,19] Dexamethasone treatment resulted in the activation of the fetal adrenal near term, which was attributed, in part, to increased fetal adrenal steroidogenic activity.[18] In addition, in females, early DEX treatment was accompanied by sex-specific fetal but not maternal changes in plasma insulin and glucose levels, suggesting that these animals were insulin resistant.[20] A suppressed response of adrenocorticotropic hormone (ACTH) but an increased ratio of cortisol to ACTH after a neuroendocrine challenge in female offsprings indicated that early DEX treatment can produce enduring effects on the hypothalamic–pituitary–adrenal (HPA) axis and its responsiveness later in life.[19,21] The HPA development and activity is associated with increased levels of ACTH and adrenal corticosteroids in the circulation of sheep and human fetuses that may be implicated in determining gestation length and producing pathophysiologic adjustments in later life.[22,23] Since the placenta is the conduit between the maternal and fetal environment, early DEX treatment at the starting point of rapid placental growth in sheep[24] may influence placental development and function and may play a role in mediating fetal GC exposure.[23,25-33]

The sheep has a synepitheliochorial, noninvasive placenta,[34] compared to hemochorial placentation in primates. The ovine cotyledonary placenta consists of placentomes that have been classified previously according to gross morphological appearance into 4 types (A, B, C, and D), reflecting the degree of eversion of the hemophagous zone.[35] Placental lactogen (PL), a member of the growth hormone (GH) family, is associated with the regulation of maternal carbohydrate, lipid, and protein metabolism.[36] In the fetus, PL may influence fetal growth indirectly through alterations in the maternal metabolic environment, maternal placental nutrient transfer to the fetus, or through stimulation of insulin-like growth factor release.[37,38] Placental lactogen is found in humans and sheep but produced by different trophoblast cell types. In sheep, ovine PL (oPL) is produced by binucleate cells (BNCs).[39] Antenatal betamethasone exposure late in gestation reduced the mean number of BNCs, reduced placental oPL-protein and maternal and fetal oPL-plasma levels as well as lowered birth weight.[26] Binucleate cells are formed from 2 uninucleate cells which, after a period of maturation, migrate through the fetal–maternal placental interface to fuse with the maternal epithelium.[40,41] The observed decrease in BNC number after GC treatment or after the rise in endogenous cortisol near term may result from an increased rate of BNC migration across the fetal–maternal interface,[42] prevention of the usual increase in BNC numbers during pregnancy,[26] or from inhibition of BNC formation and/or an imbalance of survival and apoptotic factors resulting in an increased rate of BNC apoptosis.[26,42] Glucocorticoid-induced apoptosis has been implicated in the generation of the immune response repertoire and clinically in the therapy of lymphoid malignancies.[43,44] Two pathways, the extrinsic pathway, dependent on the ligand binding to a ''death signal'' receptor (*FAS*), and the intrinsic pathway, regulated by the members of the B-cell lymphoma 2 (*BCL2*) family, consisting of pro- (*Bax*) and antiapoptotic (*BCL2*) proteins and mitochondria-derived proteins, trigger apoptosis (Figure S1).[45] Glucocorticoid might directly induce apoptosis by regulating components of either the extrinsic or intrinsic pathway or both. The fine balance between survival factors (proliferating cell nuclear antigen [*PCNA*]) and apoptosis with the activation of caspases (*Caspase-3*), as central initiators and executioners of apoptosis,[46,47] may determine placental function. It is not known whether DEX treatment in early pregnancy is associated with changes in BNC numbers and function and whether placental apoptosis is involved.

We hypothesized that DEX treatment in early pregnancy (early DEX) would alter placental development and function and therefore contribute to the previously reported immediate and long-term changes in HPA development observed after DEX treatment. Further, these effects could be sex specific.[18-21,48] To address this, we investigated changes in BNC localization and distribution, placental oPL-protein, maternal and fetal oPL-plasma levels, and placental apoptotic markers at 4 different gestational ages to determine the effect and interaction of early DEX treatment, fetal sex, and placentome subtype.

Materials and Methods

All experimental procedures were approved by the Animal Experimentation Ethics Committee of the University of Western Australia and/or the Western Australian Department of Agriculture. Briefly, pregnant Merino ewes (*Ovis aries*) with singleton pregnancies (total n = 105) of known gestational age were randomized to control (2 mL saline/ewe) or DEX-treated groups (im injections of 0.14 mg/kg ewe weight per 12 hours over 48 hours) at 40 and 41 dG as described previously.[18] Hysterectomy was performed at 49 to 51 (50), 101 to 103 (100), 125 to 127 (125), and 140 to 142 (140) dG, and placentomes were dissected from the uterus.[26] The number and weights of all placentomes per animal were recorded, and 1 representative placentome of each available subtype was randomly collected from each pregnancy. Changes in organ weights have been reported previously.[18,19]

Immunohistochemical Localization and Quantification of BNCs

Saggital cross-sections (6 μm) were taken in the middle of the placentomes. A monoclonal rabbit antibody against oPL (1:20.000 dilution, rabbit antihuman) was used as described previously.[26] Semi-quantitative analyses were performed using computerized image analysis (ImagePro Plus 4.5; Media Cybernetics, Silver Spring, Maryland).[26] A total of 12 random fields of view within 3 levels, L1-3, referring to previously described zones in a placentome,[26] were counted in each section of immunostained tissue at a magnification of 20×. A counting frame was used and BNCs were counted in an area of 0.75 μm^2. Binucleate cell with less than 30% cytoplasm

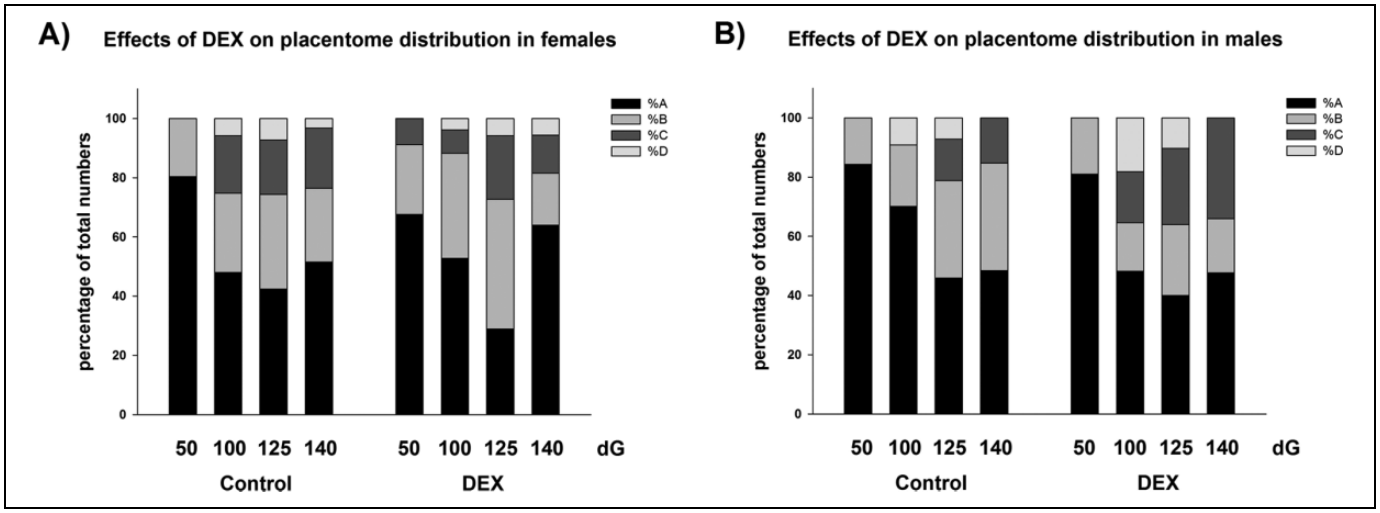

Figure 1. (A-B) The effect of early DEX treatment on placentome distribution as percentage of total numbers of placentomes in (A) females and (B) males. Data were analyzed by a full factorial model (MANOVA) with treatment, gender, and dG as type as factors, followed by a pairwise comparison (Holm Sidak) when main effects were $P < .05$. Data are presented as mean $\pm$ standard error of the mean (SEM). In females, the proportion of A subtypes placentomes in controls was lowest at 125 dG, whereas the proportion of B and C subtypes was highest at 125 dG ($P < .05$). This was similar in the DEX groups (MANOVA main effects: dG $P < .001$, type $P < .05$; interaction: dG $\times$ type $P < .05$). In males, the highest proportion of A subtypes was found at 50 dG, whereas the proportion of B subtypes was highest at 125 dG ($P < .05$). At 125 dG, DEX increased significantly the proportion of C subtypes compared to controls ($P < .05$). DEX indicates dexamethasone; dG, days of gestation.

visible were excluded. At least 4 sections per placentome (placentomes n = 204 from 105 sheep) were counted (average coefficient of variation [CV] = 7.2%). The mean number of BNCs was expressed per 0.750 mm^2 $\pm$ standard error of the mean (SEM).

Quantification of oPL-Protein Levels: Western Blotting

Quantification of oPL-protein levels with Western blotting was performed as described previously.[26] Briefly, samples from different treatment groups, sex, placentome subtypes, and days of gestation were run together on 1 gel to facilitate comparison between all factors. In total, n = 106 control and n = 111 DEX-treated placentomes were analyzed. Each blot was repeated at least 3 times with the same samples, each run 3 times. Membranes were incubated overnight with blocking solution (7.5% skim milk powder in phosphate-buffered saline-Tween) and then overnight with the same primary antibody as used for immunohistochemistry, but at a dilution of 1:80.000 for oPL. All blots were reincubated with anti-β-actin (*ACTB*) 1:20.000 (107K4800; Sigma, Sigma- Aldrich Chemie Gmbh, Eschenstrasse 5, Munich, Germany) as an internal control to allow correction for gel loading and transfer. The oPL-protein was identified as a doublet at 22 kDa (upper band = first band and lower band = second band; Figure 1C). The intensity of both oPL bands and *ACTB* was quantified by densitometry using Quantity One 4.6.2 (Bio-Rad, Bio-Rad Laboratories GmbH, Heidemannstrasse, Munich, Germany). Results were expressed as the ratio of protein to *ACTB* as relative optical density (ROD) $\pm$ SEM (average CV = 6.4%). As described previously, placental oPL-protein was identified as 2 close bands at 22 and 23 kDa with no other background signal.[26] Both bands were analyzed together (mean) as well as each band separately (upper band = first and lower band = second band; Figure 1).

Quantification of oPL-Plasma Levels: Radioimmunoassay

Concentrations of oPL-plasma were measured using equilibrium radioimmunoassay as described previously and validated in sheep.[37] There was no significant cross-reaction with ovine PRL, GH, follicle-stimulating hormone, lutenizing hormone, or thyroid-stimulating hormone.[49] The minimal detectable dose was 0.1 ng/mL, the intra-assay CV was 9.8%, and the interassay CV was 16%. Values are expressed in terms of recombinant oPL (M3RD86; Gentech, Arcade, JY).

Quantification of Placental Proliferation-, Pro-, and Antiapoptotic Markers: Quantitative Polymerase Chain Reaction

Total placenta RNA was extracted using the RNeasy Midi kit (QIAGEN, Australia) and stored at −80°C until further use. For quantitative polymerase chain reaction (q-PCR), primer pairs for sheep (Table 1) were either designed (*ACTB*, *Caspase-3*, *PCNA*, *p53*, and *FAS*) using Primer 6.0 (PRIMER-E Ltd, United Kingdom) according to the manufacture's manual or have been reported previously (ribosomal protein, large P0 [*RPLP0*],[50] hypoxanthine phosphoribosyltransferase 1 [*HPRT1*],[51] *Bax*,[52] and *BCL-2*[53]). The q-PCR assays were run on an ABI 7500 Real Time PCR System (Applied Biosystems, Life Technologies GmbH, Darmstadt, Germany). Primer

Table 1. Primer Used for Quantitative RT-PCR in Sheep Placenta and Cycling Conditions.[a]

Gene	Primer Sequences (5′ → 3′)	Product Size, bp	Efficiency, %	Annealing Temp, °C	Denaturation and Extension, °C	Cycles	Accession Number
RPLP0	F: CAA CCC TGA AGT GCT TGA CAT R: AGG CAG ATG GAT CAG CCA	227	95.9	60	95, 72	40	NM_001012682
ACTB	F: CAT CGG CAA TGA GCG GTT CC R: CCG TGT TGG CGT AGA GGT	146	97.75	60	95, 72	40	NM_001009784
HPRT1	F: GCT GAG GAT TTG GAG AAG GTG T R: GGC CAC CCA TCT CCT TCA T	94	97.6	60	95, 72	40	NM_001034035.1
PCNA	F: GCTGTTACCATAGAGATGAATG R: ATACTGAGTGTTACTGTAGGAG	107	99.9	57.2	95, 72	40	AF416380
Caspase-3	F: TCTTCAGAGGGGACTGTTGC R: ACTTTGAGTTTCGCCAGGAA	206	99.9	60	95, 72	40	AF068837.1
BCL-2	F: TTCGCCGAGATGTCCAGcC R: TTGACGCTCTCCACACACATG	155	96.5	63	95, 72	40	DQ152929.1
Bax	F: CAG GAT GCA TCC ACC AAG AAG C R: TTG AAG TTG CCG TCG GAA AAC ATT	164	95.4	60	95, 72	40	AF163774
p53	F: GAAGAATCGCAGGCAGAA R: CTCGGAGGACAGAAGGTT	102	96.93	61.8	95, 72	35	FJ855223.1
FAS	F: CGTGGCTGGTATCAACTC R: ACACATTCTGGCATATCTCC	168	95.5	59	95, 72	37	NM-001123003

Abbreviations: RPLP0, ribosomal protein, large, P0; ACTB, β-actin; HPRT, hypoxanthine phosphoribosyltransferase; PCNA, proliferating nuclear cell antigen; BCL-2, B-cell lymphoma 2; Bax, proapoptotic Bcl-2-family protein; p53, tumor protein 53; FASR, FAS receptor; PCR, polymerase chain reaction; temp, temperature.

[a]Efficiencies of PCR were determined using the formula $(10^{-1/\text{slope}} - 1) \times 100\%$ according to the manufacture's manual.

sequences have been tested and verified with fluorescent color band sequencing (Seqlab; Sequence Laboratories, Germany). All samples for each gene were run in triplicate. To determine the reliability of internal control genes, the average expression stability values (M) of 4 ICGs (*HPRT1*, *ACTB*, *RPLP0*, *18S ribosomal RNA*) were analyzed in all samples with geNorm Visual basic application (V 3.5; Biogazelle NV, Belgium) according to the manufacture's manual and the procedures described by Vandesompele et al.[54] Stepwise elimination of successive genes showed that *HPRT1*, *ACTB*, and *RPLP0* were the 3 most stable housekeeping genes to be used (pairwise variation in V3/4 = 0.135). Rescaled normalized expression levels of target genes were calculated according to the manufacture's manual and as described previously.[54]

Statistical Analyses

Analyses were performed by using SPSS 20 statistical software (SPSS Inc, Chicago, Illinois). Data were analyzed first for normality and equal variance (Levene test). Data that were not normally distributed were log transformed to achieve normality. Total placentome numbers and the mean numbers of each subtype were calculated for each gestational age for each treatment group. To determine treatment, dG, gender, and placentome subtype (where applicable) effects as well as an interaction between them, data sets were analyzed using a full factorial model (multivariate analysis of variance [MANOVA]) with treatment, gender, dG, and placentome subtype as factors, followed by a pairwise comparison (Holm Sidak) when main effects were $P < .05$. Main effects and interactions are indicated in Result section as well as in the figure legend when significant ($P < .05$), post hoc P values (Holm-Sidak) are indicated in figures. Data are presented as mean ± SEM. The relationship between the number of BNCs, placental oPL-protein, or oPL-plasma levels with fetal and placental weights and placental gene expression as combined data of placentome subtypes across gestation was assessed by correlation analysis (Pearson).

Results

The Effect of DEX on Placental Weight, Placentome Numbers, and Fetal Anthropometrics

In *females*, DEX did not affect total placenta weight or total placentome numbers (Table 2). In control animals, the proportion A subtypes was lowest at 125 dG, whereas the proportion of B and C subtypes was highest at 125 dG ($P < .05$; Figure 2A). Dexamethasone did no significantly change this distribution (MANOVA main effects: dG $P < .05$, type $P < .05$; interactions: dG × type $P < .05$). Mean placentome weight in *females* after DEX treatment was only different from controls in C subtypes at 100 and 140 dG (MANOVA main effects: dG $P < .001$, type $P < .001$; interactions: gender × type $P < .05$, dG × gender × type × treatment $P < .05$; Table 2). As reported previously,[18,19] DEX significantly reduced fetal weight in *females* at 100 dG (control 888 g ± 23.4 vs DEX = 853 g ± 52.3; MANOVA main effects: dG $P < .05$, gender

Table 2. Total Placental Weight, Mean Placentome Weight, and Placentome Numbers.[a]

	Total		A		B		C		D	
Days of Gestation	Weight, Mean ± SEM	Numbers, Mean ± SEM	Weight, Mean ± SEM	Numbers, Mean ± SEM	Weight, Mean ± SEM	Numbers, Mean ± SEM	Weight, Mean ± SEM	Numbers, Mean ± SEM	Weight, Mean ± SEM	Numbers, Mean ± SEM
(A) Placentome type in females										
50										
Control	61 ± 9.3a	59 ± 4.6a, c	1.0 ± 0.14a	56.2 ± 4.3a	1.2[b]	16[b]	NA	NA	NA	NA
DEX	40 ± 10.3A	47 ± 5.1A	0.9 ± 0.29A	44.7 ± 5.7A	1.2[b]	16[b]	NA	NA	NA	NA
100										
Contro	473 ± 35.8b	73 ± 2.2b	5.8 ± 0.68b	56.9 ± 7.4a	8.2 ± 1.52a	19.4 ± 5.8a	**8.8 ± 4.03**a	13.3 ± 9.9a	8.8 ± 4.04a	6.7 ± 4.0
DEX	431 ± 32.0B	60 ± 8.1A	5.0 ± 1.03A, B	38.6 ± 11.1A	8.6 ± 1.55A, B	17.6 ± 6.9A	**22.4 ± 3.25**A	3.2 ± 1.6A	10.3 ± 1.95	2.0 ± 1.0
125										
Control	418 ± 40.9b	57 ± 4.6c	4.5 ± 0.65a, b	29.2 ± 12.8b	7.5 ± 0.67a	25.3 ± 4.5a	8.9 ± 1.9a	15.0 ± 2.8a	8.6 ± 1.52a	4.7[b]
DEX	423 ± 46.2B	60 ± 5.1A	5.4 ± 0.85A, B	20.0 ± 9.0A	4.8 ± 1.20A	33.7 ± 8.9A	12.0 ± 5.4B	12.0 ± 5.4A	7.3[b]	5.0[b]
140										
Control	499 ± 58.2b	70 ± 6.0a, b, c	6.1 ± 0.49b	52.8 ± 11.3a	8.7 ± 1.11a	18.8 ± 4.4a	**12.8 ± 3.03**a	10.8 ± 4.8a	18.4[b]	3.0[b]
DEX	513 ± 29.0B	60 ± 6.8A	7.7 ± 1.87B	44.5 ± 5.2A	11.7 ± 3.12B	13.0 ± 3.1A	**18.8 ± 4.16**A	6.0 ± 4.0A	1.9[b]	5.0[b]
(B) Placentome type in males										
50										
Control	69 ± 7.2a	52 ± 3.8a	1.3 ± 0.09a	50.2 ± 3.5a	2.6 ± 0.12a	10.5 ± 2.5a	NA	NA	NA	NA
DEX	58 ± 9.0A	47 ± 3.9A	1.0 ± 0.16A	43.6 ± 4.5A	2.7 ± 0.46A	8.4 ± 1.9A	NA	NA	NA	NA
100										
Control	501 ± 55.3b	75 ± 4.9b	9.9 ± 1.18b, c	68.6 ± 5.7a	5.9[b]	22.0[b]	NA	NA	7.7[b]	9.0[b]
DEX	396 ± 26.1B	65 ± 6.4B	5.2 ± 0.42B	43.5 ± 9.1A	7.7 ± 1.28B	15.8 ± 3.0B	10.6 ± 3.74A	8.5 ± 3.3A	9.9 ± 2.10A	10.5 ± 4.9A
125										
Control	404 ± 33.6b	76 ± 7.1b	4.9 ± 0.25a, c	40.5 ± 11.5a	5.8 ± 0.68a	27.0 ± 8.0a	9.4 ± 3.36a	10.5 ± 5.7a	3.2[b]	9.0[b]
DEX	466 ± 42.3B	80 ± 2.8C	5.8 ± 0.64B	42.0 ± 17.7A	6.1 ± 0.94A, B	30.0 ± 7.6A, B	5.5 ± 0.72B	33.4 ± 6.2B	8.1 ± 0.71A	9.2 ± 4.0A
140										
Control	577 ± 46.1b	71 ± 6.0b	6.4 ± 0.63b, c	38.1 ± 10.3a	**6.5.0 ± 1.5**a	25.6 ± 8.8a	13.3 ± 1.49a	13.7 ± 3.7a	NA	NA
DEX	571 ± 62.9B	59 ± 6.9A, B	9.2 ± 1.77B	35.8 ± 8.1A	**12.7 ± 2.20**C	14.5 ± 3.8A, B	12.7 ± 4.42A	21.5 ± 11.2A, B	NA	NA

Abbreviations: DEX, dexamethasone; MANOVA, multivariate analysis of variance; NA, no placentomes available; SEM, standard error of the mean.

[a]Data were analyzed with a full factorial model (MANOVA) with dG, gender, placentome subtype, and treatment as factors followed by a pairwise comparison (Holm Sidak) when main effects were $P < .05$. Data are presented as mean ± SEM per sheep. Placentome weight MANOVA main effects: dG $P < .001$, type $P < .001$; interactions: sex × type $P = .027$, dG × sex × type × treatment $P = .009$; placentome numbers MANOVA main effects: type $P < .001$; interaction: dG × type $P = .025$. Different small letters (a–c) indicate significant results ($P < .05$) of post hoc analysis (Holm Sidak) across gestation in controls. Different capital letters (A–C) indicate significant results ($P < .05$) of post hoc analysis (Holm Sidak) across gestation in DEX. Significant differences between control versus DEX are indicated in bold.

[b]Total number of placentomes is small, other statistics not reported.

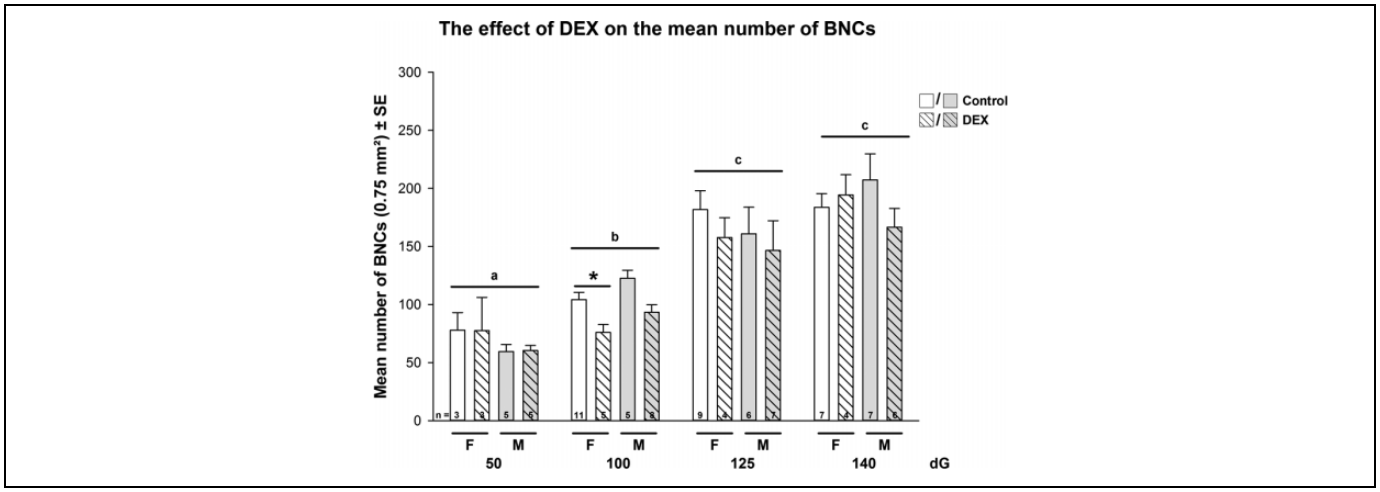

Figure 2. The effect of early dexamethasone (DEX) treatment on the mean number of binucleate cells (BNCs) in sheep placentomes during pregnancy (combined data of placentome subtypes). Data were analyzed by a full factorial model (MANOVA) with treatment, gender, and dG as factors, followed by a pairwise comparison (Holm Sidak) when main effects were $P < .05$. Data are presented as mean $\pm$ standard error of the mean (SEM) per sheep. MANOVA main effects: age $P < .001$, treatment $P < .05$; interaction: dG $\times$ treatment $P < .05$. Post hoc P values <.05 are indicated in figure: different letters indicate significant differences in dG, the star indicates significant differences in treatment. n = numbers of animals analyzed.

$P < .05$; interaction: dG $\times$ treatment $P < .05$; Holm-Sidak post hoc analysis $P = .035$), but weight was restored to normal at 125 dG (control 2787 g $\pm$ 116.8 vs DEX 2694 g $\pm$ 143.6; $P > .05$).[18,19] Crown–rump length was significantly reduced at 100 dG in *females* after DEX compared to controls (control 36.4 cm $\pm$ 0.8 vs DEX = 33.5 cm $\pm$ 0.8; MANOVA main effects: dG $P < .05$, treatment $P < .05$; interaction: $P > .05$; Holm-Sidak Post hoc analysis $P = .009$; Table 3).

In *males*, DEX did not affect total placenta weight or total placentome numbers (Table 2). The highest proportion of A subtypes was found at 50 dG, whereas the proportion of B subtypes was highest at 125 dG (Figure 2B). The DEX treatment significantly increased the proportion of C subtypes at 125 dG compared to controls ($P < .05$). Mean placentome weight in *males* after DEX treatment was only different from controls in B subtypes at 140 dG (MANOVA main effects: dG $P < .001$, type $P < .001$; interactions: gender $\times$ type $P < .05$, dG $\times$ gender $\times$ type $\times$ treatment $P < .05$; Holm-Sidak post hoc analysis $P = .03$, Table 2). Analysis of D subtypes was difficult and due to the low numbers, the values are not reported. No significant differences in fetal weight, crown–rump length, abdominal circumference, or ponderal index were observed in *males*. Only femur length at 140 dG was significantly reduced after DEX compared to controls (control 12.7 $\pm$ 0.2 cm vs DEX = 11.8 $\pm$ 0.3 cm; MANOVA main effects: dG $P < .05$, gender $P < .05$, treatment $P < .05$; interaction: $P > .05$; Holm-Sidak post hoc analysis $P = .005$; Table 3).

Overall, the ratio of placental to fetal weight as a reflection of placental efficiency in controls was not significantly different between *males* and *females*, and DEX did not affect the ratio significantly (ANOVA main effects: dG $P > .05$, treatment $P > .05$; interaction $P > .05$).

The Effect of DEX on BNCs

In control groups, the mean number of BNCs increased significantly between 50 and 125 dG, thereafter mean numbers of BNCs did not change toward 140 dG (MANOVA main effects: dG $P < .001$, type $P < .05$, treatment $P < .05$; interaction: dG $\times$ treatment $P < .05$, dG $\times$ type $P < .05$; Figure 3). Dexamethasone decreased significantly in *females*, the mean number of BNCs compared to controls at 100 dG (Figure 3), predominantly seen in A subtypes ($P < .05$). The same trend was observed in *males*, although not significant ($P > .05$).

The Effect of DEX on Placental oPL-Protein Levels

In controls, mean oPL-protein level increased significantly between 50 and 100 dG in both *females* and *males* but did not change significantly later in pregnancy (MANOVA main effects: dG $P < .001$; interaction: dG $\times$ gender $\times$ treatment $P < .05$; Holm-Sidak post hoc analysis $P < .05$). The level of first band oPL-protein increased significantly between 50 and 100 dG, but significantly decreased afterward (MANOVA main effects: dG $P < .001$, treatment $P < .05$; interaction: dG $\times$ gender $P < .05$; dG $\times$ gender $\times$ treatment $P < .05$; gender $\times$ type $P < .05$; Holm-Sidak post hoc analysis $P < .001$, Figure 1A). The level of second band oPL-protein increased significantly between 50 and 140 dG (MANOVA main effects: dG $P < .001$; interactions: dG $\times$ gender $P < .05$; dG $\times$ treatment $P < .05$, gender $\times$ type $P < .05$; Holm-Sidak post hoc analysis $P < .05$, Figure 1B). The ratio of oPL first to second band in controls was highest at 100 dG and decreased significantly toward 140 dG (MANOVA main effects: dG $P < .001$; interactions: gender $\times$ treatment $P < .05$; Holm-Sidak post hoc analysis $P < .05$, Table 3).

Table 3. Summary of the Effects of Early DEX Treatment on Fetal and Placental Development in Sheep and the Role of Placental Apoptosis.[a]

	50 dG			100 dG			125 dG			140 dG		
	Females	♀ vs ♂	Males	Females	♀ vs ♂	Males	Females	♀ vs ♂	Males	Females	♀ vs ♂	Males
Anthropometrics												
Weight	ns	Ns	ns	DEX < Con	<(DEX)	ns	ns	ns	ns	ns	ns	ns
Crown–rump length	Ns	Ns	ns	DEX < Con	ns	ns	ns	ns	ns	ns	ns	ns
Abdominal circum.	Ns	Ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
Femur	NA	NA	NA	ns	ns	ns	ns	ns	ns	ns	<(Con)	DEX < CON
Ponderal index	Ns	ns	Ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
Placenta												
Total wt	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
Placentome wt	ns	ns	ns	DEX > Con[C]	ns	ns	ns	ns	ns	DEX > Con[C]	ns	DEX > Con[B]
Placentome numbers	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
BNC numbers	ns	ns	ns	DEX < Con[A]	ns	ns	ns	>(DEX)[B]	ns	ns	ns	ns
oPL protein												
Mean band	ns	>(Con)	DEX > Con	ns	ns	ns	ns	<(Con)	ns	DEX < Con	ns	ns
First band	ns	>(Con)[A]	DEX > Con[A]	ns	ns	DEX < Con[A]	ns	<(Con)	ns	DEX < Con[C]	ns	DEX < Con
Second band	ns	<(Con)[B]	DEX > Con[A]	ns	<(Con)[A]	ns	DEX > Con[B]	<(Con)[A, D]	ns	DEX < Con[D]	>(Con)[B, C]	ns
Ratio ½ band	ns	<(Con)	ns	DEX > Con	<(Con)	DEX < Con	DEX < Con	ns	ns	ns	>(DEX)	ns
oPL-plasma levels												
Maternal	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
Fetal	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
Antiapoptotic												
PCNA mRNA	ns	ns	ns	DEX < CON	ns	ns	ns	>(DEX)	ns	ns	ns	ns
BCL-2 mRNA	ns	<(Con)	ns	ns	ns	ns	ns	<(DEX)	ns	ns	ns	ns
Proapoptotic												
FAS mRNA	DEX < CON	ns	ns	ns	>(DEX)[D]	ns	ns	ns	ns	ns	ns	ns
Caspase-3 mRNA	ns	ns	ns	DEX > Con	ns	ns	ns	ns	ns	ns	ns	ns
Bax mRNA	ns	>(Con)	ns	DEX < Con[A]	ns	ns	DEX > Con[D]	ns	ns	ns	ns	ns
p53 mRNA	ns	<(Con)	ns	DEX < Con[A, B, C]	Ns	ns	DEX > Con[D]	ns	ns	ns	ns	ns

Abbreviations: ns, not statistically significant; NA, not available; Con, control, DEX, dexamethasone; oPL, ovine placental lactogen; mRNA, messenger RNA; PCNA, proliferating cell nuclear antigen; BCL-2, B-cell lymphoma 2.

[a]Changes in fetal anthropometric parameters, placenta weight and placental BNC numbers, oPL-protein levels, and anti- and proapoptotic markers as well as maternal and fetal oPL-plasma levels were analyzed with respect to days of gestation (dG), sex, and treatment as well as placentome types (A, B, C, and D) where applicable. In the male and female columns, the greater than sign indicates that the measured parameter in the DEX group is bigger compared to controls; the greater than sign in the gender columns indicates significant differences between males and females in each group; only significant results ($P < .05$) of post hoc analysis (Holm Sidak) are presented. Capital letter indicates placentome types: A = A types, B = B types, C = C types, and D = D types.

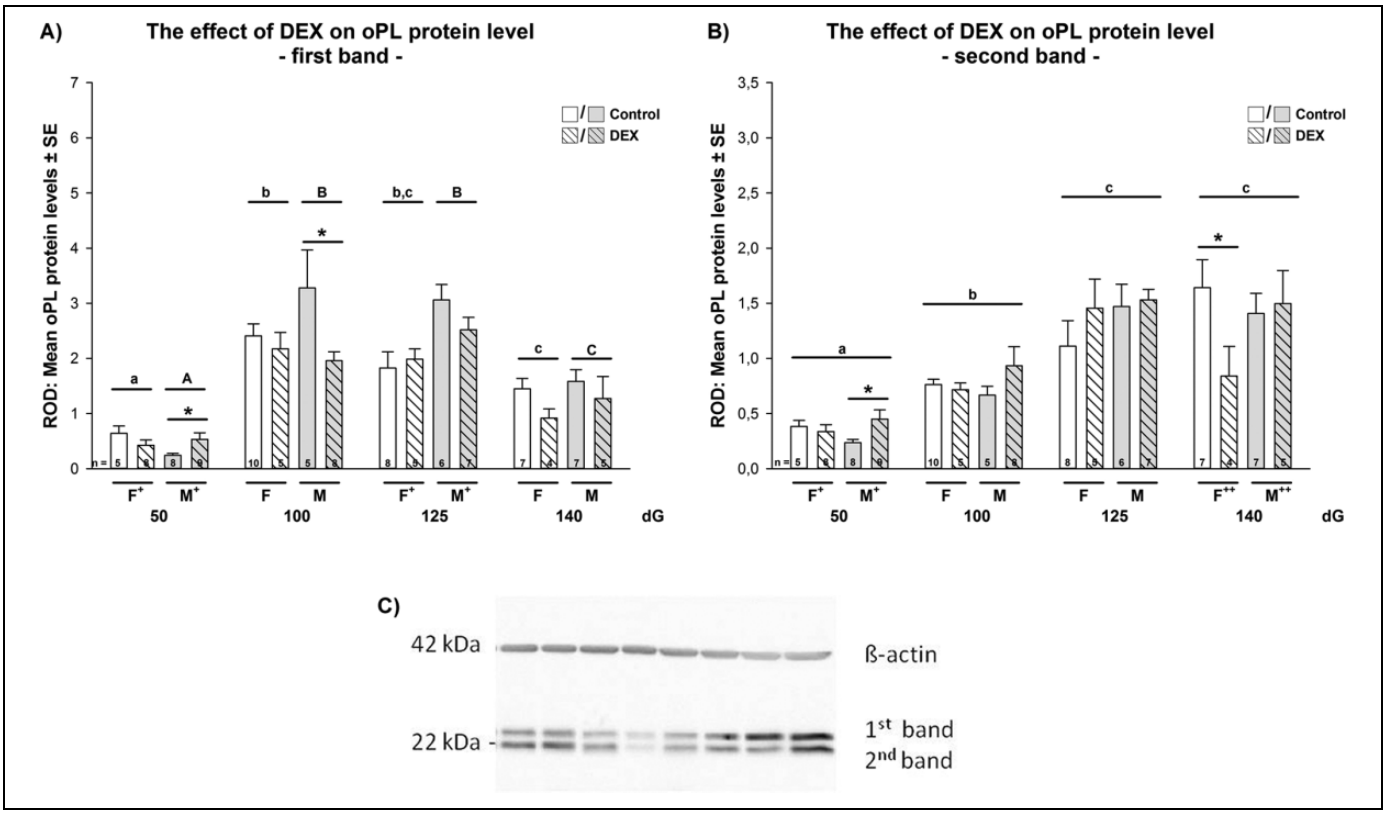

Figure 3. (A-C) The effect of early dexamethasone (DEX) treatment on the mean placental oPL-protein level in sheep. Relative optical density *(ROD) of mean placental oPL-protein level of (A) first band and (B) second band analyzed by full factorial model (MANOVA) with treatment, gender, and dG as factors, followed by a pairwise comparison (Holm Sidak) when main effects were $P < .05$. oPL first band main effects: dG $P < .001$, treatment $P < .05$; interaction: dG × gender $P < .05$; dG × gender × treatment $P < .05$; gender × type $P < .05$; oPL second band main effects: dG $P < .001$; interactions: dG × gender $P < .05$; dG × treatment $P < .05$. Post hoc P values<.05 are indicated in figure: different letters indicate sig. differences in dG, the star indicates significant differences in treatment. + represents post hoc gender differences female versus male in controls, ++ in DEX. n = numbers of animals analyzed.

Dexamethasone in *females* significantly increased oPL second band protein levels at 125 dG (mainly in B subtypes) and significantly decreased oPL second band protein levels at 140 dG compared to controls (mean, first mainly in C types and second band mainly in D types; $P < .05$; Table 3 and Figure 1). Dexamethasone significantly increased the ratio of oPL first to second band at 100 dG but significantly decreased the ratio at 125 dG compared to controls ($P < .05$, Table 3). In *males*, DEX increased significantly oPL-protein levels (mean, first and second bands) compared to controls at 50 dG mainly in A subtypes ($P < .05$; Table 3 and Figure 1). At 100 and 140 dG, oPL-protein levels in A subtypes (100 dG: first band and first/second band ratio; 140 dG: first band) were significantly decreased compared to controls ($P < .05$; Table 3).

The Effect of DEX on oPL-Plasma Level

In controls, maternal oPL-plasma levels increased significantly across gestation with highest levels at 140 dG and no effect of fetal sex (MANOVA main effects: dG $P < .001$; interactions $P > .05$; Holm-Sidak post hoc analysis $P < .05$, Figure 4A). Fetal oPL-plasma levels in controls increased significantly between 50 and 100 dG but did not change thereafter, regardless of fetal sex (MANOVA main effects: dG $P < .05$; interactions: $P > .05$; Holm-Sidak post hoc analysis $P < .05$, Figure 4B). Dexamethasone did not significantly affect maternal or fetal oPL-plasma levels in either sex.

The Effect of Early DEX on Markers of Placental Apoptosis

Details on the ontogeny of placental apoptotic markers are shown in Supplement S2. In *females*, DEX significantly reduced the antiapototic marker *PCNA* at 100 dG compared to controls (MANOVA main effects: dG $P < .05$; interactions: dG × treatment $P < .05$, dG × gender × treatment $P < .05$; Holm-Sidak post hoc analysis $P = .022$; Table 3 and Figure S2A). The proapoptotic marker *Caspase-3* was significantly increased at 100 dG compared to controls (MANOVA main effects: dG $P < .001$, treatment $P < .05$; interaction $P > .05$; Holm-Sidak post hoc analysis $P = .021$; Table 3 and Figure S2D). Although DEX induced an decrease in *BAX* (A type) and p53 (A, B, and C types) mRNA expression levels at 100 dG as compared to controls, at 125 dG, DEX significantly increased

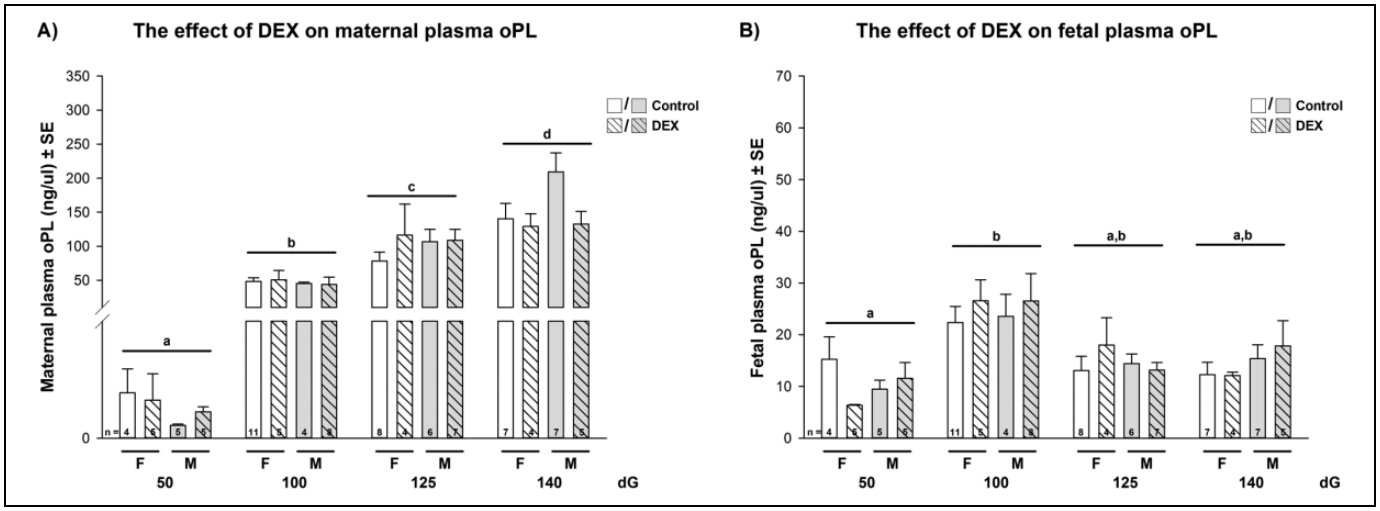

Figure 4. (A and B) The effect of early dexamethasone treatment on the mean maternal (A) and fetal (B) oPL-plasma levels in sheep analyzed by a full factorial model (MANOVA) with treatment, gender, and dG as factors, followed by a pairwise comparison (Holm Sidak) when main effects were $P < .05$. Maternal oPL main effects: dG $P < .001$; interactions $P > .05$; fetal oPL main effects: dG $P < .05$; interactions: $P > .05$. Post hoc P values <.05 (Holm-Sidak) are indicated in the figures: different letters indicate significant differences in dG.

those proapoptotic markers in D types (BAX-MANOVA main effects: dG $P < .05$, treatment $P < .05$, type $P < .05$; interactions: dG × treatment $P < .05$, treatment × type $P < .05$, dG × treatment × type $P < .05$; Holm-Sidak post hoc analysis $P < .05$; p53-MANOVA main effects: dG $P < .05$, treatment $P < .05$, type $P < .05$; interactions: dG × treatment $P < .05$; Holm-Sidak post hoc analysis $P < .05$, Table 3). In *males*, DEX did not significantly affect the measured placental apoptotic markers (Table 3).

The Relationship of BNC Numbers, Placental oPL-Protein, and Plasma Levels With Apoptotic Markers and Fetal and Placental Weight

A detailed description of significant correlations between the parameters analyzed is presented in Table S1. Briefly, in control *females*, fetal weight was positively correlated with placenta weight, number of BNCs, oPL-protein levels, and maternal oPL-plasma levels and negatively correlated with BCL-2 and FAS mRNA expression levels (Table S1A). After DEX, fetal weight changes were independent of placental oPL-protein expression levels (Table S1A). In *males*, fetal weight significantly correlated in controls with placenta weight and placentome numbers, BNC numbers, placental oPL-protein levels, and maternal oPL-plasma levels and was negatively correlated with *PCNA*, *BCL-2*, and *FAS* mRNA expression levels (Table S1B). After DEX, fetal weight changes were independent of placentome numbers (Table S1B).

Discussion

This study demonstrated that early DEX treatment is associated with sex-specific alterations in BNC numbers, and, in females, it may be associated with altered placental apoptosis markers, which may contribute to changes in placental and fetal development. The data presented may suggest that the early maternal DEX treatment in sheep, being associated with sex-specific alterations in placental development, BNC numbers, and function, may contribute to the short- and long-term changes in the fetal growth and endocrine axis observed after treatment itself.[18-21,55]

Fetal growth and development heavily depends on the capacity of the placenta during pregnancy to adapt continuously its function according to fetal demands. Fetal weight correlates strongly with placenta weight,[56] as confirmed in the present study. Early exposure to DEX resulted in a sex-dependent decrease in fetal weight and altered placental development, some of which persisted until term.[27,57] The distribution and the size of an individual placentome subtype can be influenced by adverse intrauterine conditions[58-61] and the presence of an increased number of C and D subtypes has been suggested as a placental adaptation aimed at increasing nutrient delivery to a compromised fetus.[62] We have previously shown that GC treatment late in gestation resulted in increased mean number and proportion of A subtypes and decreased numbers of B subtypes at 116 dG in males compared to controls.[27] In contrast, in the present study, the proportion of C subtypes (at 125 dG) and mean B subtype weights (at 140 dG) in males was significantly increased after early DEX treatment compared to controls. There is little information about the functional differences between placentome subtypes, but an increased proportion of everted C and D subtypes may have implications for fetal metabolism.[58,60,63-65] We have shown that changed proportions of subtypes and differential expression of important placental enzymes (prostaglandin G/H synthase 2) occurred after GC treatment in late pregnancy.[27] Early DEX treatment in females in the current study did not change placenta weight or numbers of placentome subtypes

significantly compared to controls but increased mean weights of C subtype placentomes compared to controls and may be indicative of placental adaptation to early DEX treatment.

Unique to the ruminant placenta, the trophoectoderm produces BNCs from ~14 dG and after cell maturation and migration to the fetal–maternal placental interface, BNCs fuse with the maternal epithelium[66] to form the maternal–fetal syncytium.[67] Binucleate cells account for 10% to 20% of the cells of fetal trophectoderm in sheep,[68] and their main function is to deliver fetal hormones (PLs) and effectors (pregnancy associated glycoproteins and prolactin [PRL]-related proteins) to adjust the maternal intrauterine environment to favor the needs of the fetus.[67] Ovine PL plays an important role in fetal growth through its actions on maternal metabolism and by regulating fetal substrate availability.[36] The oPL containing granules are transferred across the fetal–maternal placental interface and released into both the maternal and fetal circulation.[69] Variation in fetal weight has been correlated with placental weight, maternal serum oPL, and cotyledonary oPL mRNA concentrations.[39,70] In sheep, GC exposure late in gestation resulted in significantly lower birth weights that were associated with a reduction in the mean number of BNCs, placental oPL-protein, and maternal and fetal oPL-plasma levels.[26] In the present study, early DEX resulted in a sex-specific, transient decrease in fetal weight and crown–rump length at 100 dG in female fetuses only.[18,19] This decrease in fetal weight in females was associated with significantly lower BNC numbers but was not reflected in changes in placental oPL-protein levels. The underlying mechanism mediating the decrease in BNCs and the oPL output after DEX treatment remain to be elucidated, but an imbalance of pro- and antiapoptotic factors resulting in an increased rate of BNC apoptosis may be involved.[26,42] Indeed, placental mRNA expression levels of proapoptotic (*Caspase-3* at 100 dG, *Bax* and *p53* at 125 dG) markers were significantly increased and antiapoptotic markers (*PCNA* at 100 dG) were significantly decreased compared to controls, suggesting an activation of the placental intrinsic apoptosis pathway in females. Glucocorticoid-induced apoptosis may not critically depend on extrinsic pathways.

We recognize that our study is not without limitations. Whole placental homogenates may have masked changes that occurred in protein or mRNA levels—future studies might attempt to separate caruncles and cotyledons. However, this is difficult due to the extensive interdigitation of maternal and fetal tissue, and whole placentome staining may provide a better indication of changes in both fetal and maternal tissue.

Our data on placental oPL-protein levels permit us to relate the effects of DEX on BNC number to their function. For the first time, we were able to analyze the ROD of both oPL-protein bands separately, indicative of a glycosylated and nonglycosylated form.[26] Glycosylation is a common posttranslational modification of hormones in the PRL gene family and PL produced during the first half of pregnancy in the mouse, rat, and hamster all appear to be glycosylated.[71] Glycosylated forms of PRL have been isolated from the sheep, pig, and human and the receptor binding and biological activities of glycosylated versus nonglycosylated PRLs can be markedly different with decreased binding activities of the glycosylated forms.[71] In bovine, it has been suggested that glycosylation of PL may have a small effect on receptor specificity but does not dramatically affect receptor binding or biological activity.[71] We are not aware of published information concerning glycosylation of ovine PL. The usual gestation–dependent rise in fetal cortisol was associated with decreased placental first band (=glycosylated) oPL-protein levels between 100 and 140 dG, whereas second band (nongylcosylated) oPL-protein levels increased significantly across gestation, suggesting a decrease in glycosylation across gestation. We suggest that the lack of association between fetal weight and placental oPL-protein levels at 100 dG indicates that DEX may have disrupted the normal relationship in the maternal–fetal–placental units. At 125 dG, fetal weight and crown–rump length in DEX groups in females was restored to normal and was associated with normalized mean number of BNCs and significantly increased placental oPL-protein levels.

Fetal weight in males was significantly correlated in both controls and after early DEX with placenta weight, BNC numbers, placental oPL-protein, and maternal oPL-plasma levels. In males, early DEX treatment did not alter significantly the growth trajectory compared to controls. However at 50 dG, placental oPL-protein levels were significantly increased compared to controls (most prominent in A subtypes), which may indicate an immediate response/stimulation of BNC output. No changes in pro- and antiapoptotic markers were observed, which may be indicative of a protective placental adaptation in males.

Sex-specific adaptations to adverse maternal environments such as antenatal GC treatment have been found in animal and human studies and remain to be explained.[72-80] While male fetuses appear to adapt their placental function to maintain continued growth, female fetuses exhibit reduced growth in what is hypothesized to be an attempt to survive any further potential maternal insults.[80,81] Consistent with this, we found that male fetal weight was unaffected by early DEX treatment. Females, in contrast, exhibited temporal adaptations to DEX treatment, particularly with respect to placental distribution of subtypes and function, fetal HPA axis activity,[18] and postnatal endocrine responsiveness.[19,21,55,82] Differences in the distribution of placentome subtypes and/or function may contribute to these sex-specific responses to antenatal GC, and we now show that increased placental apoptosis in females may be a contributing factor. This contributing role of the placenta has also been observed in GC metabolizing enzymes which protect the fetus from high levels of endogenous cortisol[83] where in normal term pregnancies, females have higher levels of placental 11βHSD-2 activity compared to males.[84] These differences in enzyme activity may suggest that the female fetus could be exposed to lower maternally derived cortisol and thus escapes negative-feedback regulation, facilitating autonomous development of fetal HPA function.[85]

This study, to our knowledge, is the first study to show that early maternal DEX treatment in sheep was associated with sex-specific alterations in placental development, BNC

numbers, and function. These effects may contribute to the short- and long-term changes in the fetal growth and endocrine axis observed after early DEX treatment. In pregnancies with a female fetus, disruption of the fine balance between survival factors and apoptotic markers may influence placental function. The mode of action of GC in the inhibition of placental growth requires further investigation.

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Authors' Note

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Declaration of Conflicting Interests

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Supplemental Material

The online supplements are available at http://rs.sagepub.com/supplemental.

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2.1.4 Geschlechtsspezifische Unterschiede - Plazentarer Glukokortikoidrezeptor

H. Shang, W. Meng, D.M. Sloboda, S. Li, L. Ehrlich, A. Plagemann, J.W. Dudenhausen, J.P. Newnham, J.R.G. Challis and T. Braun: "Effects of maternal Dexamethasone treatment early in pregnancy on glucocorticoid receptors in the ovine placenta." Reproductive Sciences 2015; 22(5): 534-544[326]

Hintergrund: Die Wirkung der GC im Gewebe wird durch intrazellulär am Zellkern lokalisierte Glukokortikoid-Rezeptoren (GR), die und als ligandenabhängige Transkriptionsfaktoren fungieren, vermittelt. Die ligandenabhängige GR_{α} Isoform stimuliert im Zielgewebe die Gen-Transkription und wird als aktive Form beschrieben. Bei der ligandenunabhängigen GR_{β} Isoform vermutet man einen hemmenden Wirkungsmechanismus auf den GRα. **Arbeitshypothese:** GC-Effekte werden mittels GR vermittelt, und die frühe maternale DEX-Therapie beeinflusst die plazentare GR Expression. **Ziel:** Untersuchung der Auswirkungen maternaler DEX-Therapie auf den GR in der Schafsplazenta. **Methode:** Trächtige Mutterschafe mit Einlingsschwangerschaften wurden randomisiert und mit Injektionen von Kochsalzlösung (2ml, n=61) oder DEX (4x0,14mg/kg Mutterschafgewicht) am Tag 40 bis 41 der Schwangerschaft (dG) behandelt. An den Tagen 50, 100, 125 und 140 dG wurde Plazentagewebe asserviert. Mittels Immunhistochemie wurden die Plazentome auf die Verteilung von GR_T und GRα untersucht, mittels Western blot die GR Proteinmenge quantifiziert. **Ergebnisse:** Die DEX-Therapie erhöhte signifikant bei den weiblichen Feten die plazentare GRT Proteinmenge am Tag 50 und 125, bei männlichen Feten fand sich eine signifikante Reduktion am Tag 125. Die GRα Proteinmenge war unbeeinflusst. Neben der üblichen uninukleären Lokalisation von GRα ließen sich in den Zellkernen der BNC 3 Phänotypen Anhand von GRα-Färbemuster identifizieren (++, +-, --). DEX erhöhte die Anzahl der (++) BNC und reduzierte die Anzahl der (--) BNC am Tag 140. **Diskussion/Schlussfolgerung:** Unterschiedliche Anfärbemuster lassen vermuten, dass unterschiedliche Reifezustände von BNC existieren. Diese sind nach Leveln und Schwangerschaftstagen unterschiedlich verteilt. Während es zu einem signifikanten Anstieg der „aktiven" BNC Form zwischen Tag 50 und 100 kommt, nehmen die „inaktiven" und „intermediären" BNC Formen in diesem Zeitraum ab. Zum Zeitpunkt der Geburt zeigt sich ein umgekehrtes Bild, die Anzahl der „aktiven" BNC nimmt wieder ab, die Anzahl der „inaktiven" und „intermediären" BNC Formen wieder zu. DEX in der Frühschwangerschaft unterbricht diese Entwicklung, das heißt die Anzahl der aktiven BNC blieb konstant erhöht und könnte somit ein Hinweis für einen plazentaren Kompensationsmechanismus sein, das fetale Wachstumspotenzial trotz früher DEX-Behandlung aufrecht zu erhalten.

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Effects of Maternal Dexamethasone Treatment Early in Pregnancy on Glucocorticoid Receptors in the Ovine Placenta

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Abstract

The effects of endogenous cortisol on binucleate cells (BNCs), which promote fetal growth, may be mediated by glucocorticoid receptors (GRs), and exposure to dexamethasone (DEX) in early pregnancy stages of placental development might modify this response. In this article, we have investigated the expression of GR as a determinant of these responses. Pregnant ewes carrying singleton fetuses (n = 119) were randomized to control (2 mL saline/ewe) or DEX-treated groups (intramuscular injections of 0.14 mg/kg ewe weight per 12 hours) at 40 to 41 days of gestation (dG). Placental tissue was collected at 50, 100, 125, and 140 dG. Total glucocorticoid receptor protein (GRt) was increased significantly by DEX at 50 and 125 dG in females only, but decreased in males at 125 dG as compared to controls. Glucocorticoid receptor α (GRα) protein was not changed after DEX treatment. Three BNC phenotypes were detected regarding GRα expression (++, +−, −−), DEX increased the proportion of (++) and decreased (−−) BNC at 140 dG. Effects were sex- and cell type dependent, modifying the responsiveness of the placenta to endogenous cortisol. We speculate that 3 maturational stages of BNCs exist and that the overall activity of BNCs is determined by the distribution of these 3 cell types, which may become altered through early pregnancy exposure to elevated glucocorticoids.

Keywords

dexamethasone, glucocorticoid receptor, sheep, placenta, binucleate cell

Introduction

In women at risk of early preterm birth, maternal synthetic glucocorticoid (GC) administration is an important clinical tool to reduce neonatal mortality and morbidity from respiratory distress syndrome.[1-3] In early pregnancy, antenatal GCs are used in suspected cases of congenital adrenal hyperplasia (CAH) to prevent female fetuses from virilization.[4,5] Although the long-term effects of prenatal GC exposure late in pregnancy are well documented in experimental studies, little is known about the mechanisms that regulate outcomes of early exposure. We have previously shown in sheep that early dexamethasone (DEX) treatment resulted in sex-specific altered fetal and organ weights and function, some of which persisted into later life.[6-8]

The placenta, as the conduit between the maternal and fetal environment, and its development and function may play a role in mediating fetal GC exposure, administered early in pregnancy on key phases of placental development.[9,10] Exogenous doses of maternal GCs in pregnancy are associated with sex-specific, fetal growth restriction, and structural and functional changes in the placenta, which potentially can have life-long impact on health of the affected individual.[11] Sex-specific strategies for adapting to a changed environment in utero have been described in both animal and human studies.[12-20] In our animal studies, pregnant sheep were administered an amount of DEX analogous to that used in humans in the one-third of

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pregnancy but given as a 2-day bolus, in contrast to continuous clinical treatment with GC over weeks as in cases of CAH. We have shown that early maternal DEX therapy, as a model for both CAH treatment and early maternal distress, did not lead to growth restriction in male fetuses, whereas in female fetuses DEX treatment resulted in a transient growth reduction which was associated with significantly lower binucleate cell (BNC) numbers and increased apoptotic markers, but was not reflected in changes in ovine placental lactogen (oPL) protein levels.[21] The underlying mechanisms remain to be elucidated and the purpose of this study is to evaluate the presence of GR as an essential intermediate in regulating BNC function and placental development with respect to DEX treatment in early pregnancy.

In the sheep placenta, about 15% to 20% of the total fetal trophoblast cell number consists of BNCs. These contain granules of oPL which promote fetal growth.[22] Early DEX treatment at 40 to 41 days of gestation (dG) in females resulted in reduced BNC numbers, reduced placental anti-*proliferating cell nuclear antigen*, and increased proapoptotic factors (*Bax*, *p53*), associated with a temporary decrease in fetal growth.[21] However, neither placental oPL protein nor fetal or maternal plasma levels were changed at 100 dG. The regulatory factors associated with this apparent paradox at 100 dG, suggestive of increased BNC output of oPL to maintain placental and plasma concentrations, remain unclear.

Glucocorticoid effects on intrauterine tissues are mediated in part by glucocorticoid receptors (GRs).[23] In humans, the GR (often referred to as the total GR [GRt]) has 2 major isoforms, namely GRα (777 amino acids) and GRβ (742 amino acids) which share 1 to 727 amino acids and differ in their carboxy-terminal sequences as well as molecular weights.[24-26] The ligand-dependent GRα stimulates GC target gene transcription and is hypothesized to be the active receptor isoform.[25] GRβ is ligand independent and acts as a dominant negative regulator of GRα,[26] although conflicting evidence exists.[27-29] In sheep, during the onset and progression of spontaneous labor, temporal and tissue-specific patterns of GR expression within intrauterine tissues have been described earlier.[30] GRt, GRα, and GRβ proteins have been localized to trophoblast cells of the placenta, fetal amnion, chorion, and in maternal endometrium.[30] The localization did not change during labor progression, the protein levels of GRt, as well as GRα increased in placental tissues during labor, suggesting the possibility of increased responsiveness to cortisol at the onset and during progression of labor.[30]

Glucocorticoid treatment significantly affected BNC numbers and function[21,31]; however, it is not clear whether the responses in BNCs are due to the exogenous GC treatment or due to changes in the endogenous cortisol levels or due to an interaction between them at the GR level, for example, due to a DEX-induced downregulation of GR, which makes the tissue less responsive to endogenous cortisol. Therefore, the aim of this study was to investigate the effects of endogenous plasma cortisol and exogenous early maternal DEX in sheep on GR.

We investigated the effects of early DEX treatment in sheep on GR localization and distribution and measured GR protein levels in placentomes. Colocalization studies of GRα-stained BNCs with oPL as a marker for BNC function and caspase 3 as a marker for apoptotic degradation were performed to investigate GR-related BNC function. Based on our previous studies, we hypothesized that effects would be sex-specific and placentome subtype dependent.[21,32-34]

Materials and Methods

All experimental procedures were approved by the Animal Experimentation Ethics Committee of the University of Western Australia and/or the Western Australian Department of Agriculture. Pregnant Merino ewes (*Ovis aries*) with singleton pregnancies of known gestational age were randomized to control- (n = 59, 2 mL saline/ewe) or DEX-treated groups (n = 49, intramuscular injections of 0.14 mg/kg ewe weight per 12 hours over 48 hours, in total 4 injections) at 40 to 41 dG. Hysterectomy was performed at 49 to 51 (50), 101 to 103 (100), 125 to 127 (125), and 140 to 142 (140) dG, and placentomes were dissected from the uterus.[31,34] Placentomes were bisected and halves were either snap frozen in liquid nitrogen before storage at −80°C or fixed in 4% paraformaldehyde for 24 hours (Sigma Chemical Co., St. Louis, Missouri) according to standard procedures for future embedding in paraffin prior to sectioning. The placentomes that have been identified according to gross morphological appearance (4 types: A, B, C, and D), reflecting the degree of eversion of the hemophagous zone.[35] Maternal (jugular) and fetal (cardiac at 50 dG or umbilical arterial) blood samples and other major fetal organs were collected for use in other studies.[6-8,21]

Localization of GRt, GRα, GRβ, and Caspase 3

GRt, GRα and GRβ. Immunohistochemical staining of GR isoforms were performed on saggital cross-sections (6 μm) in the middle of the placentomes.[31] The specificity for GRt and GRα has been previously demonstrated[30,36] and was confirmed in this study with Western blotting (Figure S1). Briefly, incubation with monoclonal mouse antirat GRt at 1:50 (MA1-510, Thermo Scientific, Utah),[36] polyclonal rabbit antihuman GRα at 1:100 (P-20:sc1002, Santa Cruz),[30] or polyclonal rabbit antihuman GRβ at 1:100 (PA3-514, Thermo Scientific)[30,37] diluted in 2% normal goat serum was performed in a humidity chamber at 4°C overnight. Tissue sections were incubated with biotinylated antirabbit immunoglobulin G (IgG) antibody (PK-4001 Vectastain ABC kit, Vector Laboratories) at 1:200 and treated with ABC solution for 60 minutes (PK-4001 Vectastain ABC kit, Vector Laboratories California). Slides were exposed to 3′,3-diaminobenzidine tetrahydrochloride (DAB, Roth Chemical Kansas) as a chromogen for 10 minutes. Tissue sections from each group were processed in 1 assay to allow direct comparison between experiments. Three sets of negative controls were included as follows: (1) the primary antibody was substituted by nonimmune rabbit serum (1:200 dilution); (2) the

peroxidase-labeled secondary link antibody (goat antirabbit and donkey antirabbit immunoglobulin) was substituted with nonimmune goat/horse serum (1:200 dilution); and (3) the slide section was only incubated with nonimmune goat/horse serum (1:200 dilution) before the addition of the substrate–chromogen solution.

Caspase 3. Sections were incubated with polyclonal rabbit anti-human active caspase 3 at 1:1000 (AF835, R&D Systems, Australia)[38] and with biotinylated antirabbit IgG antibody at 1:200 (cat. no. 711-066-152, Jackson ImmunoResearch Europe Ltd., United Kingdom). Sections were labeled with streptavidin–alkaline phosphatase and the red precipitate was developed with Fast red (cat. no. K5005, Dako Real Detection System, Denmark).

Colocalization of GRα, oPL, and Caspase 3

Double staining of GRα and oPL. The GRa staining with DAB was performed in the first step, then the sections were washed in Tris-HCL (pH 7.5, Carl Roht GmbH and Merck AG, Germany) buffered for 5 minutes and 3 × 5 minutes in phosphate-buffered saline (PBS) before incubated with a rabbit anti-oPL at 1:20 000 and Fast red (ab64254, Abcam, UK) was used as chromogen for oPL.

Double staining of GRα and caspase 3. GRa staining with DAB was performed in the first step, then the sections were washed in Tris-HCL (pH 7.5, Carl Roht GmbH and Merck AG, Germany) buffered for 5 minutes and 3 × 5 minutes in PBS and 0.15% Tween-20 (Sigma Chemical Co.), followed by Caspase-3 staining with Fast red as described previously.

Image Analyses

Semiquantitative analyses were performed using computerized image analysis (ImageJ 1.43u, National Institutes of Health, USA). To reduce the number of false positive counts, GRα BNCs were counted only if >30% of the cytoplasm of a BNC was visible and at least 80% of the randomly selected field of view was covered with placental tissue.[31] A total of 18 random fields of view were counted in each section of immunostained tissue at a magnification of 20×. At least 2 sections per placentome (n = 191 from 97 sheep) were counted, over 6500 fields of view were analyzed by 1 person blinded to the treatment protocol. The mean number of BNCs was expressed per 1500 mm^2 ± standard error of the mean (SEM).

Quantification of GRt, GRα, and GRβ Protein Levels: Western Blotting

Quantification of protein levels with Western blotting was performed as described previously.[31] Each gel contained samples of different treatment groups, sex, placentome subtypes, and gestational age to facilitate comparison between all factors and was repeated at least 3 times. Membranes were incubated overnight with the same primary antibodies used for immunohistochemistry, but at a dilution of 1:1000 for GRt, 1:200 for GRα, and 1:200 for GRβ. The antibody–antigen complex was detected using a chemiluminescence detection system (34075, ECL, Thermo Scientific). Membranes were incubated without primary antibody or with primary antibody, preabsorbed with specific binding peptides (GRα blocking peptide: sc-1002 P, Thermo Scientific, GRβ blocking peptide: PEP-222, Thermo Scientific). Since there is no GRt blocking peptide for this commercially available specific antibody, the protein sequence of the GRt 97-kDa band was analyzed by Proteome Factory (Berlin, Germany) and the antibody specificity for GRt (GenBank:EU371026.1) was given. All blots were reincubated with anti-β-actin at 1:20 000 (107K4800, Sigma) as an internal control to allow correction for gel loading and transfer. Band density for both the protein of interest and β-actin was quantified by densitometry (Quantity One 4.6.2, Bio-Rad, California). Results were expressed as the ratio of protein to β-actin as relative optical density.

Statistical Analysis

Analyses were performed by using SPSS 20 statistical software (SPSS Inc, Chicago). Data were tested for normal distribution and equal variance (Levene test, $P > .05$). Data that were not normally distributed were log transformed to achieve normality. *GRα BNC numbers/mean percentages*: To determine dG, treatment, GRα subtypes (++, +−, −−), placentome subtype, and gender effects as well an interaction between them on GRα BNC numbers, data sets were analyzed using a full factorial model (multivariate analysis of variance [MANOVA]) with dG, treatment, GRα subtype (++, +−, −−), placentome subtype, and gender as factors. *GR protein levels:* To determine dG, treatment, placentome subtype, and gender effects as well an interaction between them on GR protein levels, data sets were analyzed using an MANOVA with dG, treatment, placentome subtype, and gender as factors. The MANOVA tests were followed by a pairwise comparison (Holm-Sidak) when main effects were $P < .05$. Main effects and interactions are indicated in the Result section as well as in Figure 1 where significant ($P < .05$), post hoc *P*-values (Holm-Sidak) are indicated. The relationship between the mean number of GRα stained BNCs with previously reported placental oPL protein and maternal and fetal oPL plasma levels[21] and maternal and fetal cortisol plasma levels[6] of the same cohort of animals (Table 1) was assessed by Pearson's correlation analysis. Data are presented as mean ± SEM. Statistical significance was accepted for values $P < .05$.

Results

Localization and Distribution of GR

As described previously, GRt and GRα were localized in the cytosol and nucleus of the trophoblast cells throughout the placentome and in fetal membranes[30,36] and GR presence was independent of gestational age, fetal sex, placentome subtype, and treatment. No specific GRβ staining was detectable. GRα

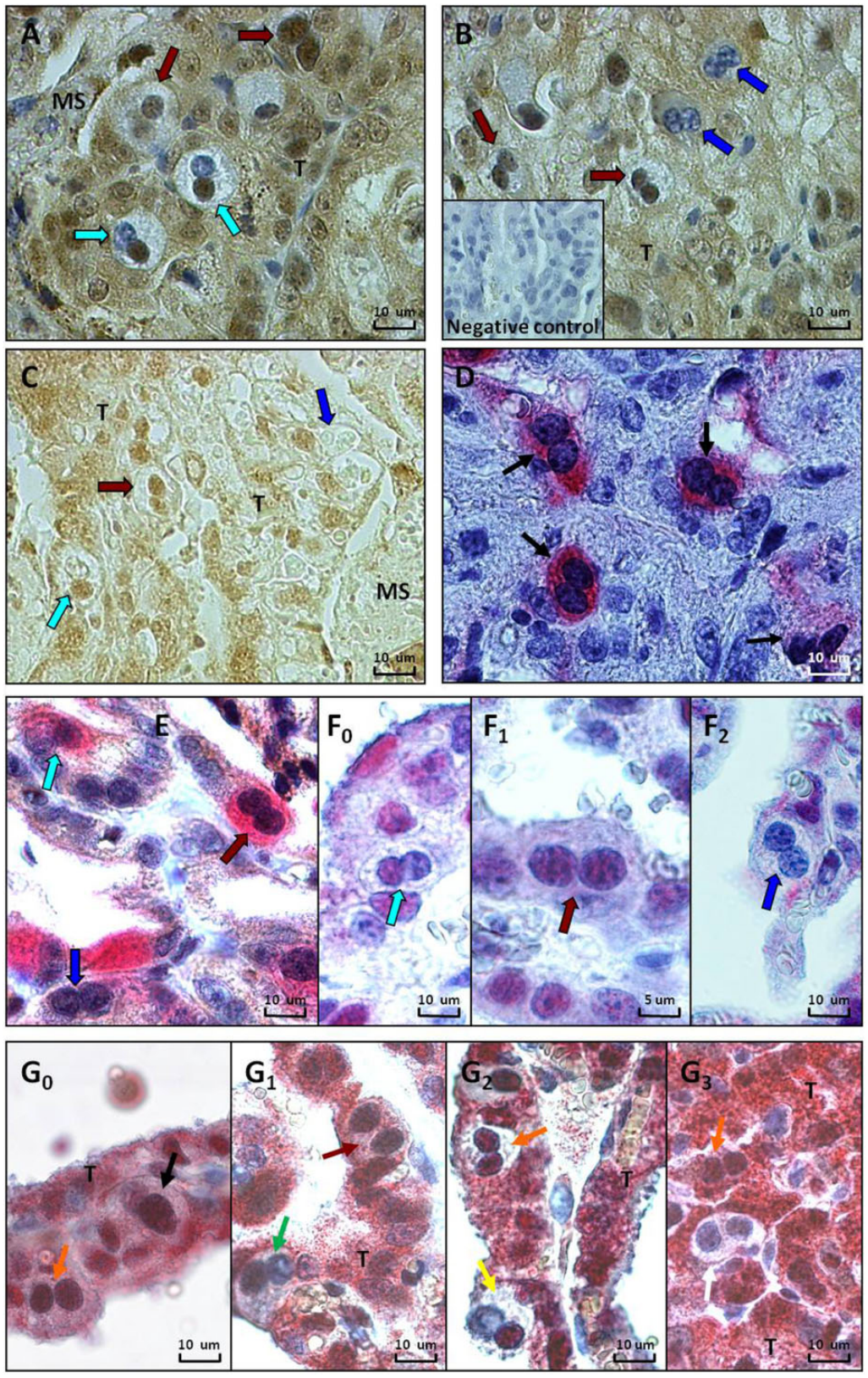

Figure 1. A-H, Immunohistochemical localization of GRα, oPL, and caspase 3 in BNCs in sheep placentomes. A and B, GRα (DAB, brown) BNC staining with hematoxylin counterstaining, 3 different nuclear staining patterns were found: BNCs with 2 brown GRα positive stained nuclei (++, red arrows), BNCs lacking any brown GRα staining in the nuclei (−−, blue arrows), and BNCs with 1 positive and 1 negative GRα staining

staining was also present within BNC, with 3 different nuclear staining patterns observed: BNCs with 2 GRα positive stained nuclei (++), BNCs lacking any GRα staining in the nuclei (−−), and BNCs with 1 positive and 1 negative stained GRα nucleus (+−; Figure 1A and B). These nuclear staining patterns were found in both males and females, control- and DEX-treated placentomes, and were independent of placentome subtype and gestational age.

Irrespective of BNC types, placentome subtype or gender, mean number of GRα BNCs in controls did not change across gestation ($P > .05$, Figure 2A). In all fetuses, (++) BNC was the most frequent cell type and (−−) the least frequent cell type (MANOVA main effects: dG $P < .05$, treatment $P < .05$, and GRα-type $P < .05$; placentome subtype $P < .05$; interaction: dG × treatment $P < .05$, dG × GRα-type $P < .05$, dG × placentome subtype $P < .05$). The mean number and proportion of (++) GRα BNCs in controls significantly increased from 50 to 100 dG and decreased thereafter from 100 to 140 dG ($P < .05$; Figure 2B). The proportion of total of (+−) and (−−) decreased from 50 to 100 dG and thereafter increased between 100 and 140 dG ($P < .05$, Figure 2B).

The Effect of DEX on GRα Localization and Distribution

Early DEX treatment significantly reduced the mean number of (++) GRα BNCs at 100 dG and the mean number of all 3 GRα BNC types at 140 dG (Figure 2A). In contrast to controls, the proportion of all 3 nuclear GRα BNC staining patterns did not change between 100 and 140 dG, therefore resulting in significant lower proportion of (−−) GRα BNCs and significant higher proportion of (++) GRα BNCs at 140 dG as compared to controls (MANOVA main effects: dG $P < .05$, treatment $P < .05$, GRα-type $P < .05$; interactions: dG × GRα-type $P < .05$, $P < .05$; dG × treatment $P < .05$; Figure 2B).

Placental GR Protein Levels and the Effect of DEX

GRt protein was detected as a single 97-kDa band as described previously[39] and the detected band was proven to be specific for GRt by protein sequencing (GenBank: EU371026.1). In control males, GRt protein levels were highest at 125 dG and decreased toward term (MANOVA main effects: dG $P < .05$; interaction: treatment × type $P < .05$; dG × treatment × sex $P < .05$; Figure 3A). In control females, no significant changes in GRt protein levels across gestation were observed. Dexamethasone significantly increased GRt protein level in females at 50 dG and at 125 dG compared to controls (Figure 3A). This decrease was significant for A-subtypes at 50 dG and for B-subtypes at 125 dG ($P < .05$). In males, however, GRt protein levels were significantly decreased at 125 dG compared to controls (Figure 3A), predominantly in C- and D subtypes.

GRα protein was identified at 95-kDa as described previously[30,36] and its specificity was proven with a blocking peptide (Figure S1A). In both males and females, lowest GRα protein levels were found at 140 dG (MANOVA main effects: dG $P < .05$; interaction: $P > .05$; Figure 3B). Dexamethasone did not affect GRα protein level ($P > .05$, Figure 3B). No differences in GRα protein levels were found regarding placentome subtypes.

The band found at 97-kDA which was described as GRβ previously[30] could not be blocked with the specific blocking peptide that is currently commercially available (Figure S1B).

The relationship between GRt (protein) and GRα (BNC numbers and protein) with placental oPL protein and maternal and fetal oPL, cortisol, and adrenocorticotropic hormone (ACTH) plasma levels is explained subsequently:

In control *females*, GRα BNC numbers (++) were negatively correlated with previously reported fetal cortisol[6] plasma levels ($r = -.446$, $P < .01$) and were positively correlated with previously reported oPL protein[21] levels ($r = .592$, $P < .01$, Table S1). After DEX treatment, (++) GRα BNC types were independent of fetal cortisol plasma levels, but the (+−) GRα BNC became positively correlated with oPL protein and maternal oPL plasma levels ($P < .05$). The GRt protein levels lost their positive correlation with oPL protein levels after DEX, whereas GRα protein levels became positively correlated with oPL protein levels ($r = .481$, $P < .05$) after DEX treatment. Both GRt and GRα protein levels did not significantly correlate with maternal or fetal cortisol and ACTH plasma levels.

In *males*, GRα BNCs numbers of all types were positively correlated with oPL protein levels ($P < .05$). After DEX treatment, this was not seen anymore for (+−) and (−−) GRα BNCs (Table S1). GRα BNCs and protein levels were independent of maternal or fetal cortisol levels ($P > .05$), but after DEX treatment, GRα protein levels were inversely correlated with fetal cortisol levels ($r = -.487$, $P < .05$). GRt protein levels in both controls and DEX were independent of maternal or fetal cortisol and ACTH plasma levels.

Figure 1. (Continued) nucleus (+−, light blue arrow). C, GRα (DAB, brown) BNC staining without hematoxylin counterstaining. D, oPL (Fast red, red) cytoplasma staining in BNCs. E, GRα (DAB, brown) and oPL (Fast red, red) BNC double staining: BNCs with oPL positive stained red cytoplasma and 2 GRα positive stained brown nuclei (red arrows); BNCs with oPL positive red stained cytoplasma and 1 positive brown and 1 negative blue GRα stained nucleus (light blue arrow) and BNCs without oPL red positive stained cytoplasma and no GRα brown stained nucleus (blue arrows). (F0-F2) Caspase 3 (Fast red, red) BNC nucleus staining: BNCs with 2 red caspase-3 positive stained nuclei (red arrow), BNCs lacking any red caspase-3 staining in the nuclei (blue arrow), and BNCs with 1 positive and 1 negative caspase-3 staining nucleus (light blue arrow). (G0-G3) caspase 3 (Fast red, red) and GRα (DAB, brown) BNC double staining: BNCs with 2 GRα positive brown nuclei lacking of caspase-3 staining (red arrow), BNCs with 1 positive GRα brown stained nucleus and 1 positive caspase-3 stained red nucleus (black arrow), BNCs with 2 positive caspase-3 stained red nuclei lacking of GRα staining (orange arrow), BNCs with 1 positive GRα brown stained nucleus and lacking of caspase-3 staining (green arrow), BNCs with 1 positive caspase-3 red stained nucleus and lacking of GRα staining (yellow arrow), BNCs with lacking of GRα and caspase-3 staining (white arrow). Representative negative control (inset B). T indicates trophoblast; MS, maternal syncytium; BNCs, binucleate cells; GR, glucocorticoid receptor; oPL, ovine placental lactogen; DAB, 3′,3-diaminobenzidine tetrahydrochloride.

Table 1. Effects of Early DEX Treatment on Fetal Development in Sheep.[a]

	50 dG				100 dG				125 dG				140 dG			
	Females		Males		Females		Males		Females		Males		Females		Males	
	CON, n = 6	DEX, n = 6	CON, n = 10	DEX, n = 10	CON, n = 11	DEX, n = 5	CON, n = 5	DEX, n = 8	CON, n = 9	DEX, n = 4	CON, n = 6	DEX, n = 7	CON, n = 7	DEX, n = 4	CON, n = 7	DEX, n = 6
Fetal weight,[6] g	13.7 ± 0.9	14.2 ± 0.5	14.6 ± 0.7	15.4 ± 0.5	888 ± 23.4*[1]	853 ± 52.3*[2]	958 ± 47.1[1]	936 ± 61[2]	2787 ± 117	2694 ± 144	2988 ± 86	2876 ± 178	4681 ± 122[1]	4511 ± 198	5169 ± 136[1]	4848 ± 199
Placental oPL protein,[12] ROD	0.51 ± 0.09[1]	0.38 ± 0.07	0.24 ± 0.03*[1]	0.49 ± 0.10*	1.59 ± 0.12	1.44 ± 0.17	1.97 ± 0.33	1.44 ± 0.13	1.47 ± 0.25[1]	1.72 ± 0.15	2.26 ± 0.23[1]	2.02 ± 0.12	1.59 ± 0.21*	0.88 ± 0.22*	1.49 ± 0.18	1.38 ± 0.33
Plasma oPL,[12] ng/mL																
Maternal	0.77 ± 0.41	0.65 ± 0.45	0.22 ± 0.02	0.45 ± 0.09	48.2 ± 5.4	50.6 ± 13.7	45.2 ± 1.9	43.9 ± 10.7	78.1 ± 13.3	116.4 ± 45.4	106.7 ± 18.2	108.9 ± 15.9	140.4 ± 22.4	129.2 ± 18.7	209.1 ± 28.0	132.8 ± 18.3
Fetal	15.27 ± 4.3	6.35 ± 0.15	9.45 ± 1.8	11.6 ± 3.0	22.4 ± 3.1	26.6 ± 4.1	23.6 ± 4.3	26.5 ± 5.3	13.1 ± 2.7	18.0 ± 5.3	14.4 ± 1.8	13.2 ± 1.4	12.3 ± 2.4	12.1 ± 0.7	15.4 ± 2.6	17.8 ± 4.9
Plasma ACTH,[6] pg/mL																
Fetal	37.4 ± 13.7	37.1 ± 14.1	65.7 ± 13.5	61.1 ± 15.4	89.0 ± 9.0	112.4 ± 32.4	69.0 ± 3.5	70.3 ± 11.5	142.4 ± 44.4	151.5 ± 51.2	135.8 ± 36.0	100.6 ± 9.9	128.4 ± 14.0	128.9 ± 13.3	100.8 ± 7.3	135.5 ± 13.5
Plasma cortisol,[6] ng/mL																
Maternal	41.7 ± 5.6	17.9 ± 4.5	26.6 ± 4.4	27.1 ± 6.7	31.5 ± 3.9	48.4 ± 11.8	39.1 ± 9.2	26.0 ± 4.8	43.2 ± 6.5	35.2 ± 5.5	35.2 ± 7.9	40.4 ± 8.9	72.9 ± 10.6*[1]	106.3 ± 21.6*[2]	39.5 ± 9.0[1]	57.3 ± 13.7[2]
Fetal	14.8 ± 5.5[1]	5.2 ± 2.9	**8.0 ± 1.6***[1]	**4.5 ± 0.9***	3.1 ± 0.4	3.2 ± 1.0	2.4 ± 0.6	2.7 ± 0.44	6.3 ± 0.9	6.1 ± 1.2	5.6 ± 1.4	3.8 ± 0.7	**15.9 ± 0.8***[1]	**27.9 ± 6.3***[2]	**10.0 ± 1.5***[1]	**16.9 ± 2.9***[2]

Abbreviations: CON, control; DEX, dexamethasone; ROD, relative optical density; dG, days of gestation; oPL, ovine placental lactogen; ACTH, adrenocorticotropic hormone; SEM, standard error of the mean; MANOVA, multivariate analysis of variance.

[a]Changes in fetal weight, placental oPL protein levels as well as maternal and fetal oPL, ACTH, and cortisol plasma levels were analyzed with respect to dG, sex, and treatment (MANOVA, post hoc analysis of Holm-Sidak). Significant differences between control and DEX are indicated in bold with *, Gender differences in controls are indicated with 1 in DEX with 2. Data are presented as mean ± SEM. Data have been published previously, indicated by the references.

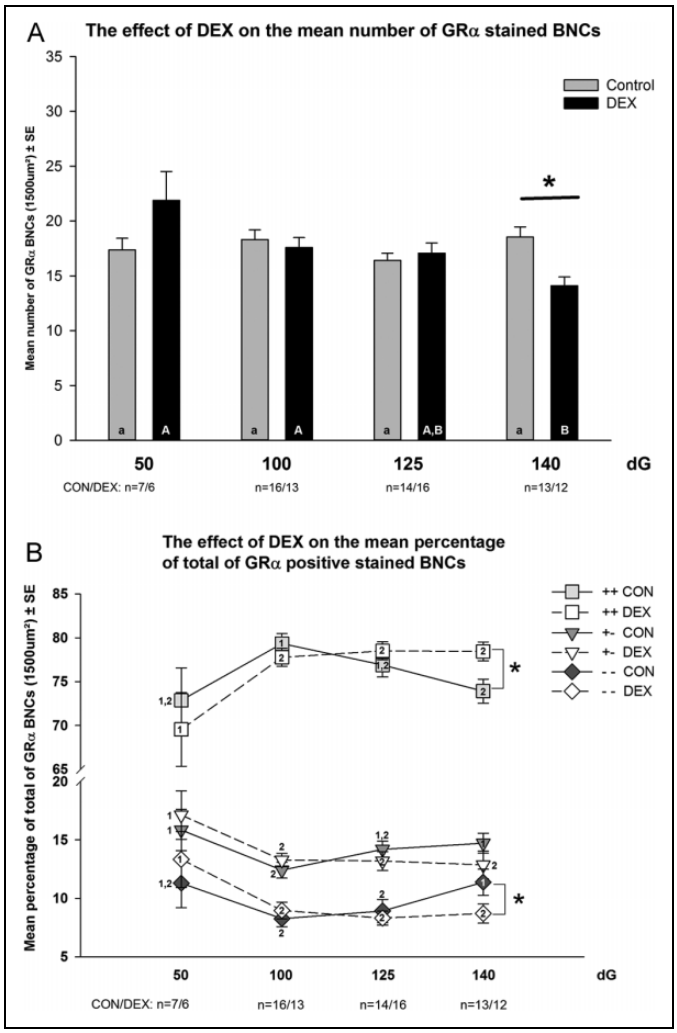

Figure 2. A, Mean number (A) and percentage (B) of GRα positive stained BNCs in control and DEX groups. A, Presenting the total number of GRα positive binucleate cells at each age, and the effect of dexamethasone, irrespective of placentome subtypes, sex, and BNC types. DEX treatment significantly reduced the mean number of GR positive stained BNCs at 140 dG*, which was significant for all 3 BNC subtypes (++, +−, −−, see Result section). Small letters (a) indicate no significant differences in control groups across gestation. Differences in capital letters indicate significant differences in the DEX groups across gestation. B, Presenting the mean percentage of total GRα positive stained BNCs at each age, and the effect of dexamethasone, irrespective of placentome subtypes and sex. Different numbers indicate significant differences across gestation per cell type. Significant differences between control and DEX per cell type are indicated with stars. The number of sheep included in the study are given subsequently (n = control/DEX). Significant difference was accepted for $P < .05$. DEX: indicates dexamethasone; BNCs, binucleate cells; GR, glucocorticoid receptors; dG, days of gestation.

Maturational Differences in BNCs: GRα Double Staining With oPL or Caspase 3

Ovine placental lactogen localized predominately to the cytoplasm of the BNCs, with little immunostaining in the maternal syncytium as reported previously (Figure 1D).[31,39] Double staining of BNCs for GRα and oPL showed 3 different staining patterns: BNCs with oPL positive stained cytoplasm and 2 GRα positive stained nuclei (++); BNCs with oPL positive stained cytoplasm and 1 positive and 1 negative GRα stained nucleus (+−); and BNCs without oPL positive stained cytoplasm and no GRα-stained nucleus (−−) (Figures 1E and 4).

Caspase 3 localized predominately to the nuclei of BNCs, with little immunostaining in the maternal syncytium, fetal, and maternal stroma. Binucleate cells with 2 caspase-3 positive stained nuclei, BNCs lacking any caspase-3 staining in the nuclei, and BNCs with 1 positive and 1 negative caspase-3 stained nucleus were found (Figures 1 [F_0-F_2] and 4). Double staining in BNCs with GRα and caspase 3 showed different staining patterns (Figures 1 [G_0-G_3] and 4).

Discussion

Dexamethasone treatment in early pregnancy is associated with transient sex-specific alterations in placental development and function and fetal growth. Placental GRt protein levels were increased significantly by DEX in females at 50 and 125 dG, but decreased in males at 125 dG as compared to controls. GRα protein levels were not changed after DEX treatment. Three BNC phenotypes were detected regarding GRα expression (++, +−, and −−); DEX increased the proportion of (++) and decreased (−−) BNC at 140 dG. Effects were sex- and cell type dependent suggesting a modified responsiveness of the placenta to endogenous cortisol.

In this study, we were able to analyze GRt and GRα protein levels from early pregnancy, at 50 dG, up to 140 dG for both females and males separately. In females, GRt protein levels did not change significantly across gestation, whereas in males, highest levels of GRt protein levels were found at 125 dG, and then decreased toward 140 dG. Placental GRα protein levels were lowest at 140 dG in both females and males, suggesting a reduced placental responsiveness to near-term cortisol. Previous studies on direct cortisol infusion to the fetus resulted in increased GRα but not GRt protein levels in sheep placenta.[30] In females, early DEX significantly increased GRt protein levels at 50 and 125 dG, in males, however, DEX significantly decreased GRt protein levels at 125 dG compared to controls. Although in female fetuses a constant placental GC sensitivity is maintained, possibly in terms of a preferential survival strategy for ensuring reproductive capacity and species conservation, it seems that in male fetuses due to increased GC exposure, the placenta becomes at least temporarily GC resistant. In both females and males, GRα protein levels were not affected by early DEX, but in males became significantly correlated with oPL protein and fetal cortisol levels after DEX treatment, indicating a greater importance of GRα for cortisol responsiveness and oPL production and secretion.

As described and discussed previously by Root et al[37] who used the same GRβ antibody as described by Gupta et al,[30] we were also not able to find a specific band for GRβ. The band found at 97-kDA was very weak and could not be blocked with the specific blocking peptide that is now commercially

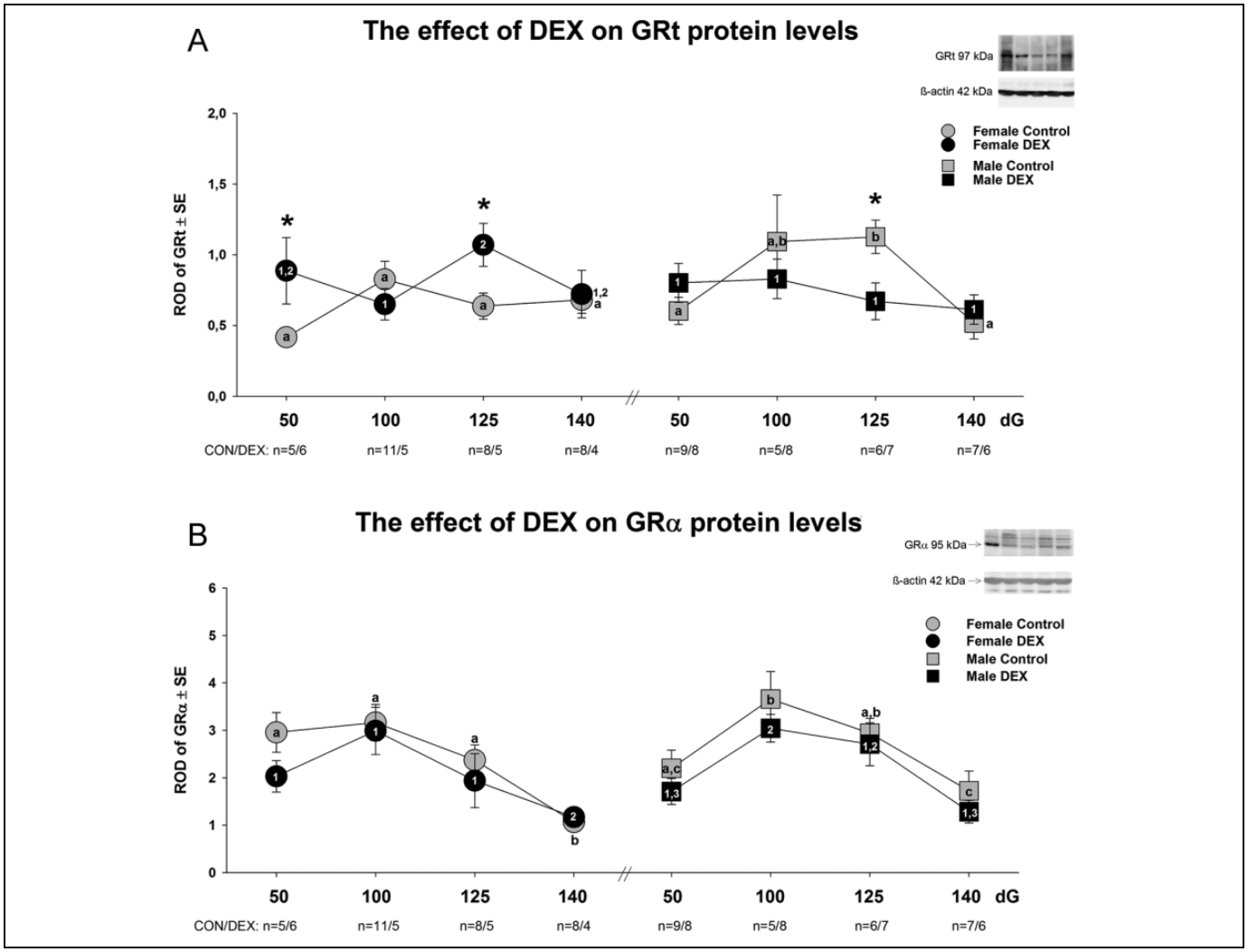

Figure 3. A and B, The effect of DEX on GRt (A) and GRα (B) protein level in males and females. Different letters (a and b) represent significant differences in control groups across gestation. Numbers indicate significant differences in the DEX group across gestation. Stars indicate significant differences between CON and DEX groups. The number of sheep included in the study are given subsequently. ROD was calculate by (GRα/β-actin)/internal control. Significant difference was accepted for $P < .05$. CON indicates control; DEX, dexamethasone; ROD, relative optical density; GR, glucocorticoid receptor.

available (Figure S1B). It was suggested that the expression of GRβ is either very low in sheep placenta or the previously reported GRβ antibody, whose specificity can now be tested with a blocking peptide, revealed a non-specific binding of GRβ.[37] In a very recent study, Saif et al identified 12 isoforms of the GR in the human placenta, which were localized to the trophoblast cells and expression varied in relation to cellular location in either the cytoplasm or nucleus, fetal sex, fetal size, but not all isoforms were expressed in every individual.[40] Therefore, we would like to suggest that other GR isoforms may also be present in the sheep placenta, which could contribute to the changes observed in GRt protein but not GRα protein, but this has to be elucidated in the future.

Evidence for differences in protein expression being affected by anatomical localization of BNCs has been reported previously.[41] Now, for the first time in this study, 3 different immunostaining patterns of GRα in BNCs have been observed. However, the separate function of these BNC types, if present, still remains unclear. We hypothesized based on our current and previous observations that the most frequently observed staining pattern, with 2 GRα positive stained nuclei (++), is a potential "active" BNC form, whereas the least frequently observed staining pattern, lacking GRα nuclear staining (−−), is an "immature" or "inactive" BNC form (Figure 4). Since BNCs produce oPL, colocalization of GRα and oPL was used to determine whether a (++) GRα BNC would contain oPL granules in the cytoplasm. Indeed, (++) GRα BNCs stained positively for oPL in the cytoplasm, whereas the (−−) GRα BNCs did not have positive oPL staining in the cytoplasm. A third GRα staining pattern in BNCs was also found, in which only 1 nucleus was positively stained for GRα, the other nucleus was lacking GRα staining (+−). Colocalization of GRα and oPL showed oPL positive cytoplasm in this third staining pattern and we speculate that this cell type may

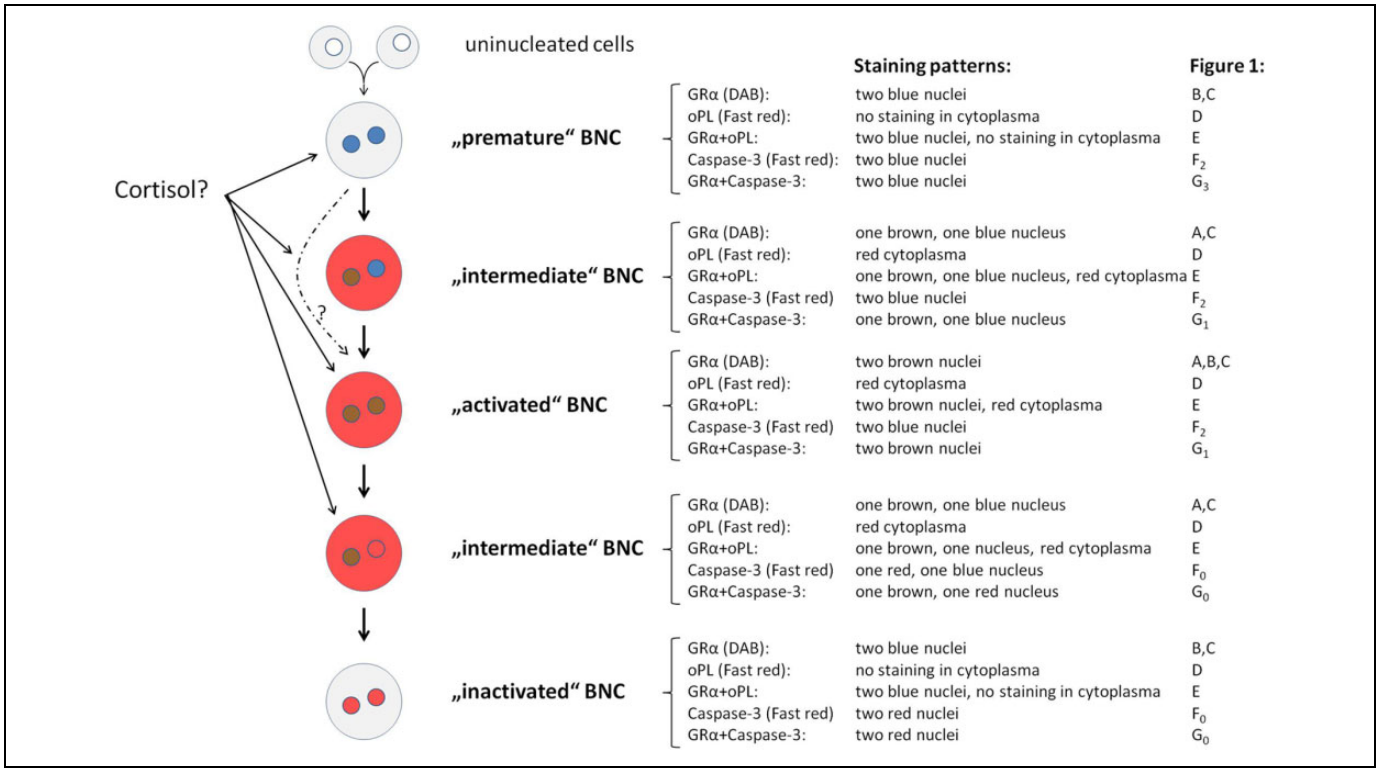

Figure 4. GRα BNC "maturation hypothesis" in sheep placenta. Interpretation of the immunohistochemical localization of GRα, oPL, and caspase 3 in BNCs in sheep placentomes of Figure 1 in relation to different "BNC activity stages." Early in pregnancy, premature GRa BNC (−−) may develop directly to activated GRα BNCs (++) since the number of intermediate BNC does not change. Later in pregnancy with increased fetal cortisol due to HPA axis activation, premature GRα BNC (−−) may develop via the intermediate GRα BNC (+−) type to the active GRα BNC (++) which is represented by an in increased number of intermediate GRα BNC (+−). BNCs indicates binucleate cells; GR, glucocorticoid receptor; oPL, ovine placental lactogen; HPA, hypothalamic–pituitary–adrenal.

be an "intermediate" BNC form. It has been shown previously by others[42-44] and also in our own DEX treatment model[21] that GC treatment can be associated with increased rates of apoptosis. Consistent with this, colocalization of GRα with active caspase 3 revealed strong nuclear caspase-3 staining in the (−−) GRα BNC to support this GRα BNC type being "inactive," whereas in the (++) GRα BNC form, no caspase-3 nuclear staining was observed. We hypothesize, that 2 maturational forms of "intermediate" (+−) GRα BNCs exist (Figure 4). The first "intermediate" GRα BNC form is present on the way from the developing "premature" BNC to an "activated" BNC without showing signs of apoptosis (negative caspase-3 staining, Figure 1 (F_2)). A second "intermediate" (+−) BNC form is present on the way from maturing from an "activated" to an "inactivated" GRα BNC, presenting signs of apoptosis (positive caspase 3, Figure 1 (F_0)).

This study was not designed to investigate the underlying mechanisms regulating the formation of different BNC types. However, the proportion of BNC staining positively for GRα in 1 or 2 nuclei changed with advancing gestational age and we hypothesize that these changes are coincident with placental maturation early in gestation and with fetal hypothalamic–pituitary–adrenal (HPA) maturation later in gestation. It has been demonstrated that BNCs develop from the fusion of 2 uninucleate trophoblast cells[22,45,46] and we may hypothesize that the different GRα-staining patterns indicate different maturational stages and function of a BNC. Although the regulatory factors that govern these processes are unknown and could not be investigated in this study, we hypothesize that cortisol itself may mediate these events and control of the production and migration of BNCs may play a pivotal role in ruminant implantation and placental growth. Early in gestation (50-100 dG), the mean number of (++) GRα BNCs increased and the number of (+−) and (−−) GRα BNCs did not change. Later in gestation (100-140 dG), as the fetal HPA axis matures and begins to secrete higher concentrations of cortisol, we observed a decrease in the mean numbers of (++) BNC resulting in an increased proportion of (+−) and (−−) GRα BNCs. Therefore, early in pregnancy, "premature" (−−) GRα BNC may develop directly to "activated" (++) GRα BNCs, since the number of "intermediate" (+−) GRα BNC did not change. Later in pregnancy with increased fetal cortisol due to HPA axis activation, "premature" (−−) GRα BNC may develop via the "intermediate" (+−) GRα BNC type to the "active" GRα BNC (++) which is represented by an increased number of "intermediate" (+−) GRα BNC (Figure 4).

Administration of DEX significantly decreased the number of (++) GRα BNCs at 100 and 140 dG compared to controls

but simultaneously changed the composition of the BNC type resulting in a relative increase in the (++) compared to (+−) and (−−) GRα BNC types. By preventing the conversion from the (++) to the (+−) and (−−) GRα BNC form, the placenta may have compensated the loss of total GRα BNCs after DEX.

Conclusion

Three different BNC phenotypes were identified, and the activity of BNCs may be determined by the distribution of these 3 cell types. Early DEX increased the proportion of BNCs from a potentially ''inactive'' to an ''active'' form, and we hypothesize that this effect may act to increase the sensitivity of GRα BNCs in near term. Effects were sex- and cell type dependent suggesting a modified and sex-specific responsiveness of the placenta to endogenous cortisol. Our understanding on how endogenous GC and/or overexposure to exogenous GC can influence fetal and placental development begins with the GR. A thorough understanding of how maternal glucocorticoid administration influences placental cellular function will provide a better understanding about the role of the placenta in the fetal adaptive response.

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Declaration of Conflicting Interests

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Supplemental Material

The online figures and tables are available at http://rs0.sagepub.com/supplemental

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2.2 Humane Studien

2.2.1 Dosisabhängige Effekte der Glukokortikoidexposition

T. Braun, D.M. Sloboda, B. Tutschek, T. Harder, J.R.G. Challis, J.W. Dudenhausen, A. Plagemann, W. Henrich: "Fetal and neonatal outcomes after betamethasone administration in term and preterm gestations" IJGO 2015; 130 (1) 64-69[116]

Hintergrund: Entsprechend den in unseren tierexperimentellen Studien am Schaf bislang gewonnen Ergebnissen zu den Auswirkungen antenataler GC-Therapie haben wir in einem translationalen Ansatz die dosis- und geschlechtsspezifischen Effekte einer antenatalen LRI mit BET bei drohender Frühgeburt auf die fetale Anthropometrie und das neonatale Outcome beim Menschen untersucht. **Arbeitshypothese:** BET-dosisabhängige Beeinträchtigung des fetalen Wachstums, die sich bereits intrauterin mit Hilfe von Ultraschall-Folgeuntersuchen nachweisen lässt. **Ziel:** Untersuchung der Auswirkungen verschiedener BET-Dosisgruppen (≤16mg, =24mg, >24mg) auf das fetale Wachstum und das neonatale Outcome im Vergleich zu altersgepaarten Kontrollen. **Methode:** Retrospektive Fallkontrollstudie an über 44.000 Schwangerschaften (BET n=1.799, Kontrollen n=42.240) an der Charité zwischen Januar 1996 und Dezember 2008. Fälle beinhalteten Frauen mit drohender Frühgeburt mit symptomatischen Wehen, Zervixinsuffizienz, vorzeitigem Blasensprung oder vaginalen Blutungen. Outcome Parameter: fetales Wachstum, neonatale Anthropometrie, umbilikale Blutgaswerte, Apgar Scores und Plazentagewicht. **Ergebnisse:** Bereits eine Einmalgabe von 2x12mg BET war mit einer signifikanten Reduktion des Geburtsgewichtes, des Kopfumfanges und der Körperlänge sowohl bei den männlichen als auch bei den weiblichen Feten im Vergleich zu den Kontrollen assoziiert. Diese Veränderungen waren auch nach multivariater Adjustierung für zahlreiche Konfounder weiterhin vorhanden. Je größer die BET-Dosis, desto stärker war der wachstumsreduzierende Effekt ((≤16mg: -444g, =24mg: -523g, >24mg: -811g), ohne dem Nachweis einer signifikanten Verbesserung der neonatalen Morbidität/Mortalität. In US-Folgeuntersuchungen konnte eine BET-dosisabhängige Abnahme des Schätzgewichtes innerhalb der ersten 6-8 Wochen nach BET-Gabe nachgewiesen werden. **Diskussion/Schlussfolgerung:** Eine BET-Dosisabhängigkeit konnte bestätigt werden, wobei eine höhere BET-Dosierung mit einer stärkeren Verringerung des Wachstums verbunden war, nicht jedoch wie bislang postuliert, mit einer verbesserten neonatalen Morbidität oder Mortalität. Im Gegenteil, höhere Dosierungen waren bei den männlichen Feten mit einer signifikanten Verschlechterung der APGAR Werte assoziiert.

Weitere eigene, relevante Publikationen zu diesem Thema:

T. Braun, A. Husar, J.R.G. Challis, J. W. Dudenhausen, W. Henrich, A. Plagemann, D. M. Sloboda: „Growth restricting effects of a single course of antenatal betamethasone treatment and the role of placental lactogen." Placenta 2013; 34: 407-415[123]

G. Justus*, D. M. Sloboda*, J. W. Dudenhausen, W. Henrich, A. Plagemann and **T. Braun**: "Avoiding Prenatal Programming Effects of Glucocorticoids: Are there Alternative Treatments for Antenatal Lung Maturation?" JPME 2015; 43(5): 503-523 *shared first co-authorship[57]

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CLINICAL ARTICLE

Fetal and neonatal outcomes after term and preterm delivery following betamethasone administration

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ABSTRACT

Objective: To determine the effects of betamethasone on fetal growth and neonatal outcomes. *Methods:* A retrospective cohort study was performed of deliveries that occurred at Charité University Hospital Berlin, Germany, between January 1996 and December 2008. The betamethasone group included women with preterm labor and symptomatic contractions, cervical insufficiency, preterm premature rupture of membranes, or vaginal bleeding. Women in the control group were matched for gestational age at time of delivery and had not received betamethasone. Fetal growth changes and neonatal anthropometry were compared. *Results:* Among 1799 newborns in the betamethasone group and 42 240 in the control group, betamethasone was associated with significantly lower birth weight (154 g lower on average) after adjusting for confounders (e.g. hypertension, smoking, and maternal weight), sex, and gestational age at delivery ($P < 0.05$). The higher the dose, the greater the difference in mean birth weight versus controls in births before 34^{+0} weeks (≤16 mg −444 g; 24 mg −523 g; >24 mg −811 g), without a detectable improvement in neonatal morbidity or mortality. There was a dose-dependent decline in expected fetal weight gain as estimated by serial ultrasonography examinations 6–8 weeks after betamethasone administration ($P < 0.05$). *Conclusion:* Betamethasone exposure reduces fetal weight gain in a dose-dependent manner without improving neonatal morbidity or mortality.

1. Introduction

Prenatal administration of synthetic glucocorticoids is a powerful intervention to reduce the frequency of respiratory distress syndrome, and neonatal morbidity and mortality among women at risk of preterm birth [1]. Until the late 1990s, repeated maternal glucocorticoid treatment was common practice for women who did not deliver within 7 days of the initial course [2]. This practice was mainly based on experimental evidence showing that surfactant production and lung maturation in fetal sheep is maximized after serial doses of glucocorticoids administered over a period of weeks [3]. Although there is evidence against weekly glucocorticoid injections [4], others support the use of repeated doses for women still at risk of preterm delivery at least 7 days after an initial course, quoting a reduced risk of adverse neonatal outcome [5,6]. Discussion is ongoing about the dose, type of steroid, treatment interval, and whether repeated doses of glucocorticoids are beneficial [7–10]. Differences in effects between the sexes, whereby male fetuses benefit less than do female fetuses, have also been suggested in animal and human studies [11].

The aim of the present study was to determine the effects of maternal betamethasone treatment in initially normally grown fetuses on fetal growth, neonatal outcome, and placental weights.

2. Materials and methods

The present retrospective cohort study comprised data collected for 44 039 deliveries at the Clinic of Obstetrics, Charité University Hospital Berlin, Germany, between January 1, 1996, and December 31, 2008 (data stored in database KIM Argus, GMT GmbH, Frankfurt, Germany). The Charité University Hospital ethics review committee approved the retrospective, observational, and anonymized analysis, and deemed that no formal ethical review or written consent from individual patients was needed.

The deliveries were divided on the basis of whether the mothers had received prenatal treatment with betamethasone. Women in the

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betamethasone group had been diagnosed with preterm labor and symptomatic contractions, cervical insufficiency, preterm premature rupture of membranes, or vaginal bleeding, and received betamethasone (Celestan, MSD GmbH, Haar, Germany) between 23 weeks plus 5 days of pregnancy (23^{+5} weeks) and 33^{+6} weeks. Controls were women with gestational-age-matched pregnancies (on the basis of time of delivery) who did not receive betamethasone treatment. The exclusion criteria were multiple gestations, major malformations, pregnancies with incomplete datasets for all variables under investigation, and pregnancies in which a glucocorticoid other than betamethasone was administered.

For women diagnosed with preterm labor, several dose regimens and intervals of betamethasone treatment were used. These regimens varied between two 8-mg doses (≤16 mg) or two 12-mg doses (24 mg) given once during pregnancy, and repetitive doses of betamethasone (8 mg weekly, or two 12-mg doses every second week) when the diagnosis of preterm labor still persisted (collectively grouped as >24 mg). For the present study, the effects of betamethasone dosage on neonatal outcomes were compared among three different total dosages: 16 mg or less, 24 mg, and more than 24 mg. A time variable (year of delivery) was introduced in the multivariate analyses to adjust for secular trends.

The estimated date of delivery was corrected by early prenatal ultrasonography when the difference between the due date by the last menstrual period and that by early sonography was more than 7 days. Fetal weight before betamethasone treatment and during follow-up ultrasonography was calculated by using the Hadlock formula. Fetuses with an estimated fetal weight (EFW) below the 10th centile at the time of the initial treatment were defined as small for gestational age and excluded from the study.

The effects of betamethasone on neonatal anthropometrics (birth weight, head circumference, body length), placental weight, ponderal index, umbilical cord blood gases (umbilical artery blood pH and umbilical vein blood pH), base excess, Apgar scores (1-, 5-, 10-minute), and neonatal mortality were analyzed separately for both sexes. Neonatal morbidity included respiratory distress syndrome or asphyxia, hypoglycemia (newborn plasma concentration <1.7 mmol/L within 24 hours of delivery), and neonatal infections requiring antibiotic treatment.

Standard curves for fetal weight in the control population were generated using LMS ChartMaker (Medical Research Council, Cambridge, UK); centiles and *Z* scores for anthropometric data were calculated with LMS Growth (Medical Research Council, Cambridge, UK). To analyze the effects of betamethasone on fetal growth, initial and follow-up EFW *Z* scores were calculated and betamethasone-associated changes were controlled for possible confounders.

Considering the periods in pregnancy clinically relevant for neonatal outcome, groups were divided by gestational age: very early preterm (group I: 25^{+0}–27^{+6} weeks; group II: 28^{+0}–30^{+6} weeks), early preterm (31^{+0}–33^{+6} weeks), late preterm (31^{+0}–36^{+6} weeks), term (37^{+0}–39^{+6} weeks), and post-term (≥40 weeks).

Effects of betamethasone treatment on neonatal body measurements, placental weight, ponderal index, umbilical cord blood gases, neonatal mortality and morbidity, and Apgar scores were analyzed using the Mann–Whitney *U* test. Sex-specific statistical analyses were performed in the whole cohort. Betamethasone effects were analyzed among three different dosages (≤16 mg, 24 mg, and >24 mg) using the Mann–Whitney *U* test. By multivariate analyses of variance, betamethasone-associated birth weight changes were controlled for possible confounders.

Data are presented as mean ± standard error. Analyses were performed by using SPSS version 20 (IBM, Armonk, NY, USA). The odds ratios (ORs) with 95% confidence intervals (CIs) for a birth weight in the 10th centile or less and Apgar scores of less than 7 were calculated by binary logistic regression using a birth weight reduction equal to or less than the 10th centile and/or an Apgar score of less than 7 as dependent variables and betamethasone dose as covariates. $P < 0.05$ was considered significant.

3. Results

The betamethasone group included 1799 newborns (979 male and 820 female) and the control group included 42 240 newborns (21 773 male and 20 467 female). The overall number of women treated with betamethasone per year was 63 (4.0%; range 42–86 [2.5%–6.0%]) among pregnancies with female fetuses and 75 (4.6%; range 45–115 [2.7%–7.6%]) among those with male fetuses. Regarding pregnancy prolongation after betamethasone exposure, 558 (68.0%) women carrying female fetuses and 734 (75.0%) women carrying male fetuses remained pregnant for longer than 7 days after treatment; 369 (45.0%) and 480 (49.0%) women, respectively, delivered after 34^{+0} weeks, and 213 (26.0%) and 274 (28.0%) women, respectively, delivered after 37^{+0} weeks. Maternal characteristics are shown in Table 1. None of the maternal variables examined accounted for anthropometric changes after treatment in multivariate analyses (Supplementary Material S1).

Standard curves for fetal weight in the control population were generated (Supplementary Material S2) and the newborns (EFW ≥10

Table 1
Maternal characteristics of the study group.[a]

Maternal characteristics	Female newborns			Male newborns		
	Control group (n = 20 467)	BMS group (n = 820)	*P* value[b]	Control group (n = 21 773)	BMS group (n = 979)	*P* value[b]
Age at delivery, y	28.8 ± 0.0	29.3 ± 0.2	0.029	28.8 ± 0.0	29.0 ± 0.2	0.322
Weight before pregnancy, kg	64.8 ± 0.1	63.8 ± 0.5	0.001	64.9 ± 0.1	63.7 ± 0.5	<0.001
Weight at delivery, kg	77.9 ± 0.1	73.7 ± 0.6	<0.001	78.4 ± 0.1	74.1 ± 0.5	<0.001
Weight gain, kg	13.2 ± 0.0	10.1 ± 0.3	<0.001	13.5 ± 0.4	10.5 ± 0.2	<0.001
Height, cm	164.1 ± 0.1	163.6 ± 0.6	0.818	163.8 ± 0.1	164.0 ± 0.4	0.167
BMI before pregnancy	23.7 ± 0.0	23.4 ± 0.2	0.001	23.8 ± 0.0	23.3 ± 0.2	<0.001
BMI at delivery	28.5 ± 0.0	27.1 ± 0.2	<0.001	28.7 ± 0.0	27.2 ± 0.2	<0.001
BMI gain	4.9 ± 0.0	3.8 ± 0.1	<0.001	4.9 ± 0.0	3.9 ± 0.1	<0.001
Disease						
Diabetes	510 (2.5)	45 (5.5)	<0.001	573 (2.6)	71 (7.2)	<0.001
Hypertensive disorders[c]	391 (1.9)	90 (11.0)	<0.001	421 (1.9)	71 (7.2)	<0.001
Country of origin						
Germany	11053 (54.0)	547 (66.7)	<0.001	11 706 (53.8)	593 (60.6)	<0.001
Turkey	4132 (20.2)	96 (11.7)	<0.001	4406 (20.2)	138 (14.1)	<0.001
Other	5282 (25.8)	177 (21.6)	0.004	5661 (26.0)	248 (25.3)	0.334

Abbreviations: BMS, betamethasone; BMI, body mass index (calculated as weight in kilograms divided by the square of height in meters).
[a] Values are given as mean ± standard error or number (percentage), unless indicated otherwise.
[b] Calculated by Mann–Whitney *U* test for maternal parameters, and Fisher exact test for maternal complications and country of origin.
[c] Hypertension and pre-eclampsia.

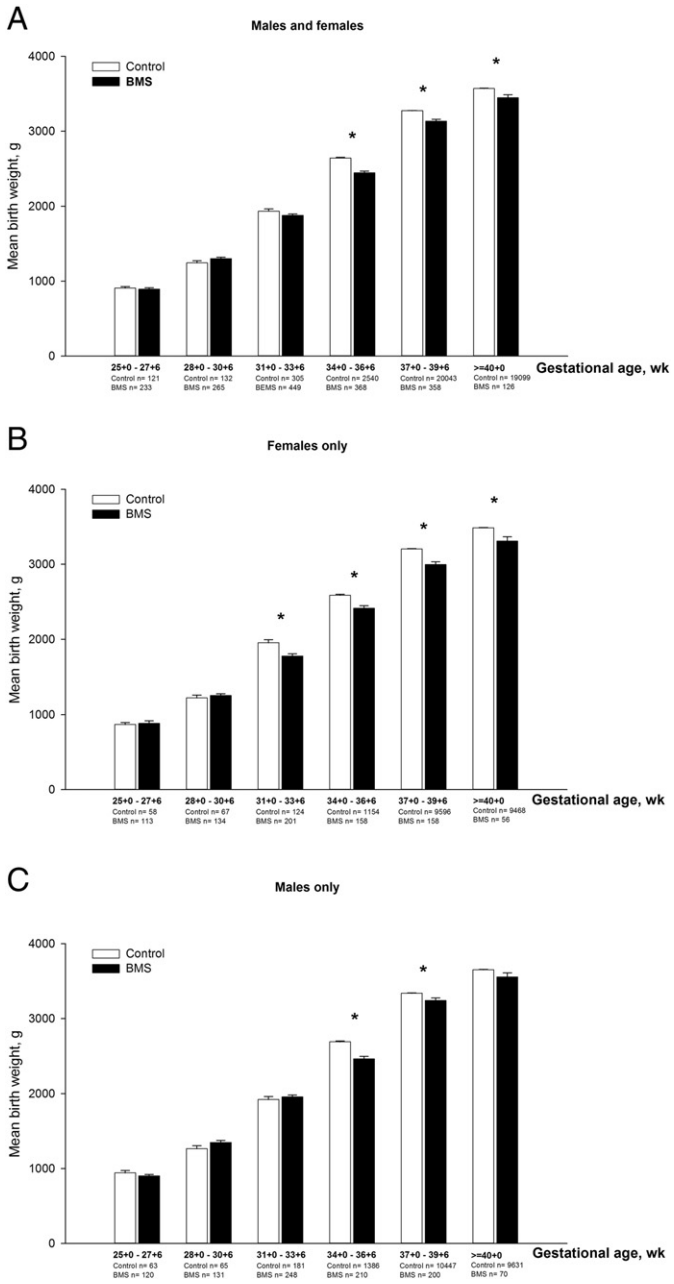

Fig. 1. Mean birth weight in the control and BMS groups. Error bars show standard errors. Asterisks indicate significant differences ($P < 0.05$). Abbreviation: BMS, betamethasone.

centile) of mothers who were exposed to betamethasone and were born late preterm, at term, and post-term had significantly lower birth weights (on average –154 g) than did age-matched controls (Fig. 1A, Table 2). When analyzing by sex, the reduction in birth weight after betamethasone treatment was significant for female neonates born after 31^{+0} weeks (Fig. 1B) and for male neonates born between 34^{+0} weeks and 39^{+6} weeks (Fig. 1C, Supplementary Material S3).

The birth weight of neonates exposed to betamethasone and born late preterm or at term was significantly lower than that of controls for all three dosage regimens analyzed (Supplementary Material S4). The difference in mean birth weight versus controls increased with betamethasone dose (Table 3). Neonates exposed to a higher dose were more likely to be on or below the 10th centile for birth weight (≤16 mg vs 24 mg: OR 1.84 [95% CI 1.01–3.35] for early preterm; OR 2.55 [95% CI 1.27–5.09] for late preterm; ≤16 mg vs >24 mg: OR 4.15 [95% CI 1.28–13.44] for term). The difference was significant only in males (Supplementary Material S5).

For 1216 fetuses, data were available to analyze the rate of fetal weight gain at the time of first exposure and 2–8 weeks after exposure. Fetal weight gain declined dose-dependently with the greatest reduction in EFW *Z* scores for fetuses treated with more than 24 mg of betamethasone (Fig. 2). The effects were independent of major confounders.

Head circumference was smaller among betamethasone-exposed females born after 34^{+0} weeks (mean −0.6 cm) and males born late preterm than among controls (mean −0.5 cm). Body length was significantly decreased among betamethasone-exposed females born after 37^{+0} weeks (mean −1.0 cm) and males born after 34^{+0} weeks (mean −0.8 cm). A significant reduction in ponderal index after exposure was found among females born early preterm (Supplementary Material S3). The betamethasone-associated reduction in head circumference and body length was independent of major confounders (Supplementary Material S1).

Overall, mean placental weight was significantly lower in the betamethasone group than in the control group (females –116 g, males –114 g) (Supplementary Material S3). Overall, Apgar scores were not significantly improved by betamethasone treatment (Table 2). Indeed, mean Apgar 1-, 5-, and 10-minute scores for the whole cohort were significantly lower in the betamethasone group than in the control group (Supplementary Material S3). For betamethasone-exposed females, no significant improvement in Apgar scores were found as compared with controls (Supplementary Material S3). For males, however, Apgar scores at 1 and 5 minutes were significantly higher in neonates born early preterm after betamethasone treatment, as were umbilical artery pH, umbilical vein pH, and base excess (Supplementary Material S3). By contrast, betamethasone-exposed males born late preterm had significantly lower 1- and 5-minute Apgar scores, and those born late preterm or at term had significantly lower 10-minute scores; betamethasone-exposed females had significantly lower 5-minute Apgar scores when born post-term (Supplementary Material S3).

Higher betamethasone dosages did not improve Apgar scores overall (Table 4). Among female neonates exposed to 24 mg of betamethasone, 1-minute Apgar scores were significantly higher among neonates born between 28^{+0} weeks and 30^{+6} weeks (group II very early preterm) than among controls (mean difference 0.9; $P = 0.048$). Females exposed to more than 24 mg betamethasone and born late preterm had decreased Apgar scores (1-, 5-, 10-minute scores: −0.8, −0.5, −0.3, respectively; $P < 0.05$). Apgar scores were significantly increased with betamethasone doses of 24 mg among male neonates born between 28^{+0} weeks and 33^{+6} weeks (1-, 5-, 10-minute scores: 1.0, 0.7, 0.4, respectively; $P < 0.05$) and among those who received up to 16 mg and were born late preterm (1- and 5-minute scores: 1.0, 0.6, respectively; $P < 0.05$). However, among males born late preterm, Apgar scores were significantly lower in betamethasone-exposed cases, independent of the betamethasone dose (1-, 5-, 10-minute scores: ≤16 mg −0.3, −0.3, −0.2, respectively; 24 mg, −0.3, −0.3, −0.2, respectively; >24 mg, −0.8 [1-minute score]; $P < 0.05$ for all).

Treatment with betamethasone significantly reduced the rates of breathing problems for neonates of both sexes born preterm (females: 51/1352 [3.6%] vs 5/601 [0.8%], $P < 0.001$; males: 71/1624 [4.2%] vs 12/697 [1.7%], $P = 0.001$). No significant differences were recorded in the rates of hypoglycemia (females: 9/1394 [0.6%] vs 10/606 [0.6%], $P > 0.05$; males: 17/1678 [1.0%] vs 4/705 (0.6%), $P > 0.05$) or infection (females: 35/1368 [2.5%] vs 11/595 [1.8%], $P > 0.05$; males: 50/1645 [2.9%] vs 14/695 [2.0%], $P > 0.05$). Betamethasone doses of more than 24 mg did not improve neonatal morbidity: the rates of breathing problems, neonatal hypoglycemia, and neonatal infections were similar to those of controls for the population as a whole (Table 4).

The proportion of neonates who died was higher in the betamethasone group than in the control group for neonates of both sexes born before 37 weeks (females: 12/1391 [0.9%] vs 19/587 [3.1%].

Table 2
Difference in neonatal parameters between control and betamethasone groups independent of sex.[a]

Fetal/neonatal outcome	Very early preterm, group I[b]		Very early preterm, group II[b]		Early preterm[b]		Late preterm[b]		Term[b]		Post-term[b]	
	Difference between groups[c]	*P* value[d]	Difference between groups[c]	*P* value[d]	Difference between groups[c]	*P* value[d]	Difference between groups[c]	*P* value[d]	Difference between groups[c]	*P* value[d]	Difference between groups[c]	*P* value[d]
Birth weight, g	–	0.672	–	0.158	–	0.233	−197	<0.001	−141	<0.001	−142	0.017
Head circumference, cm	–	0.463	–	0.998	–	0.071	−0.7	<0.001	−0.4	<0.001	−0.3	0.018
Body length, cm	–	0.407	–	0.803	–	0.181	−1.0	<0.001	−0.7	<0.001	−0.8	<0.001
Ponderal index[e]	–	0.395	–	0.419	–	0.201	–	0.348	–	0.505	–	0.197
Apgar score												
1 min	–	0.392	–	0.054	–	0.054	–	0.110	–	0.054	–	0.867
5 min	–	0.769	–	0.096	–	0.176	−0.2	0.041	−0.2	0.003	–	0.166
10 min	–	0.451	–	0.096	–	0.159	–	0.063	−0.1	0.005	–	0.606
UApH	–	0.869	–	0.265	0.03	<0.001	–	0.252	–	0.050	–	0.614
UVpH	–	0.613	–	0.134	0.03	<0.001	–	0.709	–	0.666	–	0.465
Base excess	–	0.100	–	0.085	1.31	<0.001	–	0.251	–	0.471	–	0.313
Placenta weight, g	–	0.383	–	0.079	–	0.301	–	0.060	–	0.218	–	0.527

Abbreviations: UApH, umbilical artery blood pH; UVpH, umbilical vein blood pH.
[a] Only significant differences after controlling for possible confounders by multivariate analyses of variance are shown; full data are provided in Supplementary Material S3.
[b] Across the six gestational age groups, the number of control/betamethasone neonates was 121/233, 132/265, 305/449, 2540/368, 20 043/358, and 19 099/126, respectively.
[c] Negative values indicate a reduction in the betamethasone group; positive values indicate an increase in the betamethasone group.
[d] Calculated by Mann–Whitney *U* test.
[e] 100 × birth weight (g)/body length (cm^3).

$P < 0.001$; males: 17/1678 [2.0%] vs 14/695 [1.0%], $P = 0.046$) and for females born after 37 weeks (7/19 057 [0.9%] vs 2/212 [<0.1%], $P = 0.004$). The overall increase in neonatal mortality was independent of betamethasone dosage (Table 4).

4. Discussion

In the present study, maternal betamethasone treatment was associated with lower birth weight, head circumference, and body length for both male and female neonates as compared with gestational age-matched controls, independent of major confounders, sex, or time of treatment in pregnancy. Higher betamethasone dosage regimens resulted in the greatest reduction in fetal growth without further improvement in neonatal morbidity or mortality. Fetal weight gain decreased in a dose-dependent manner in the 6–8 weeks following the initial exposure. Umbilical cord blood gas parameters were generally unchanged after betamethasone. Only among early preterm males were Apgar scores improved after betamethasone treatment. For late preterm and term pregnancies, betamethasone exposure was accompanied by significantly lower Apgar scores for both sexes.

Table 3
Effect of betamethasone dose on birth weight.[a]

Dose	Difference in birth weight, g		
	0 mg (controls)	≤16 mg	24 mg
<34^{+0} wk			
≤16 mg	−444	–	–
24 mg	−523	–	–
>24 mg	−811	−367	−288
34^{+0}–36^{+6} wk			
≤16 mg	−167	–	–
24 mg	−249	–	–
>24 mg	−293	–	–
37^{+0}–39^{+6} wk			
≤16 mg	−132	–	–
24 mg	−119	–	–
>24 mg	−464	−332	−344
≥40^{+0} wk			
≤16 mg	−139	–	–
24 mg	–	–	–
>24 mg	–	–	–

[a] Mean differences in birth weight (both females and males) are shown by gestational age at delivery and dose (betamethasone vs control, higher vs lower dose). Only data with significant differences ($P < 0.05$) are presented.

Nearly half the fetuses exposed to betamethasone treatment were born after 34^{+0} weeks, and approximately one-third were born after 37^{+0} weeks.

Birth weight is subject to a degree of constraint by the maternal intrauterine environment and a strong determinant of perinatal survival. In the present study, betamethasone treatment was accompanied by significantly lower birth weight as compared with age-matched controls, independent of major confounders. The betamethasone-induced reduction in birth weight, head circumference, and body length were independent of the duration of pregnancy after treatment. The follow-up EFW of fetuses that were initially growing normally showed that the reduction in fetal weight gain was directly related to the dose of betamethasone.

Large studies [12,13] have demonstrated that decreased birth weight, used as a surrogate marker of fetal growth and development, is associated with increased risk of adult disease. A small head circumference is strongly associated with learning difficulties among school-aged children [14]. Although the reduction in head circumference was small, the relationship between head circumference and intracranial volume is defined by a cubic function. Therefore, small reductions in head circumference are associated with a considerable decrease in intracranial volume; in the present study, the 4% decrease in head circumference observed would be associated with an 11% reduction in intracranial volume [15].

The clinically recommended dose of glucocorticoids was not empirically tested [16], and was later found to expose the fetus to concentrations comparable to physiological stress levels of cortisol that are normally present after birth [17]. The current recommended practice is one course of two 12-mg betamethasone doses for lung maturation [18,19], but repeated maternal glucocorticoid treatment was common practice until at least the late 1990s [2]. In the present study, higher doses of betamethasone resulted in a greater reduction in fetal weight gain and birth weight among both females and males. The greatest reduction in birth weight of up to 16% versus controls was observed among females who were born at term, and whose mothers had received more than 24 mg of betamethasone.

Although betamethasone significantly improved neonatal morbidity in neonates born before 37 weeks; no improvement was seen at higher dosages. In fact, mortality rates appeared to increase with higher betamethasone dosages. Only males born early preterm benefited from betamethasone treatment, with higher Apgar scores. Althabe

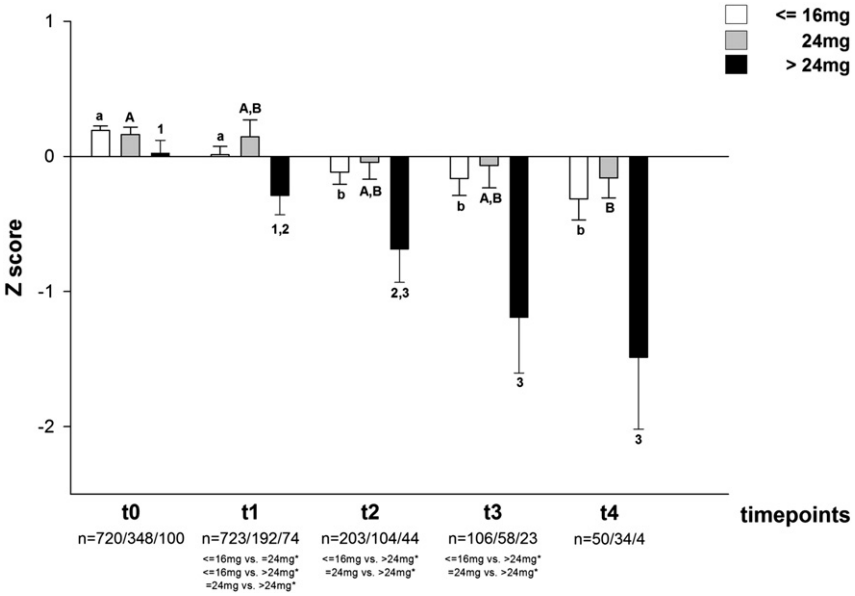

Fig. 2. Dose-dependent estimated fetal weight before and after first betamethasone treatment. t0, immediately before the first betamethasone treatment; t1–t4, follow-up ultrasonography at a mean of 18, 32, 45, and 55 days, respectively. When lower-case letters (≤16 mg), upper-case letters (24 mg), or numbers (>24 mg) are different, this indicates a significant difference between follow-up measurements ($P < 0.05$). Asterisks indicate a significant difference between doses at follow-up measurements t1–t3. Error bars show standard errors.

et al. [20] recently investigated the feasibility, effectiveness, and safety of corticosteroid treatment versus standard care for the reduction of neonatal mortality due to preterm birth in low- and middle-income countries. They raised questions regarding the uncritical use of glucocorticoid treatment. The study found that glucocorticoid intervention not only was ineffective for reducing neonatal mortality among fetuses

Table 4
Effect of betamethasone treatment on neonatal Apgar scores, morbidity, and mortality.[a,b]

Neonatal parameters	Control group	Betamethasone ≤16 mg		Betamethasone 24 mg		Betamethasone >24 mg	
		Value	*P* value[c]	Value	*P* value[c]	Value	*P* value[c]
Both sexes	42 240	1177	–	474	–	148	–
Apgar score							
1 min	8.7 ± 0.0	7.2 ± 0.1	<0.001	7.1 ± 0.1	<0.001	6.5 ± 0.2	<0.001
5 min	9.6 ± 0.0	8.4 ± 0.0	<0.001	8.5 ± 0.1	<0.001	8.1 ± 0.1	<0.001
10 min	9.8 ± 0.0	9.0 ± 0.0	<0.001	9.0 ± 0.1	<0.001	8.8 ± 0.1	<0.001
Morbidity[d]							
Breathing problems[e]	797 (1.8)	18 (1.5)	0.269	7 (1.5)	0.371	3 (2.0)	0.505
Hypoglycemia[f]	285 (0.7)	4 (0.3)	0.105	2 (0.4)	0.381	1 (0.7)	0.368
Infections[g]	460 (1.1)	24 (2.0)	0.004	1 (0.2)	0.035	4 (2.7)	0.080
Mortality[d]	52 (0.1)	20 (1.7)	<0.001	9 (1.9)	<0.001	6 (4.1)	<0.001
Females	20 467	557		209		54	
Apgar score							
1 min	8.7 ± 0.0	7.1 ± 0.1	<0.001	7.0 ± 0.1	<0.001	6.5 ± 0.3	<0.001
5 min	9.6 ± 0.0	8.3 ± 0.1	<0.001	8.4 ± 0.1	<0.001	8.3 ± 0.2	<0.001
10 min	9.8 ± 0.0	8.9 ± 0.1	<0.001	9.0 ± 0.1	<0.001	8.9 ± 0.1	<0.001
Morbidity[d]							
Breathing problems[e]	301 (1.5)	4 (0.7)	0.091	2 (1.0)	0.406	2 (3.7)	0.190
Hypoglycemia[f]	119 (0.6)	1 (0.2)	0.052	1 (0.5)	0.297	1 (1.9)	0.733
Infections[g]	180 (0.9)	13 (2.3)	0.002	1 (0.5)	0.159	1 (1.9)	0.381
Mortality[d]	19 (0.1)	15 (2.7)	<0.001	3 (1.4)	0.001	3 (5.6)	<0.001
Males	21 773	620		265		94	
Apgar score							
1 min	8.6 ± 0.0	7.3 ± 0.1	<0.001	7.2 ± 0.1	<0.001	6.4 ± 0.3	<0.001
5 min	9.6 ± 0.0	8.5 ± 0.1	<0.001	8.5 ± 0.1	<0.001	8.0 ± 0.2	<0.001
10 min	9.8 ± 0.0	9.1 ± 0.0	<0.001	9.0 ± 0.1	<0.001	8.7 ± 0.2	<0.001
Morbidity[d]							
Breathing problems[e]	466 (2.1)	14 (2.3)	0.460	5 (1.9)	0.499	1 (1.1)	0.400
Hypoglycemia[f]	119 (0.8)	4 (0.6)	0.491	2 (0.8)	0.671	1(1.1)	0.488
Infections[g]	280 (1.3)	11 (1.8)	0.186	1 (0.4)	0.146	3 (3.2)	0.123
Mortality[d]	33 (0.2)	5 (0.8)	0.004	6 (2.3)	<0.001	3 (3.2)	<0.001

[a] Values are given as number, mean ± standard error, or number (percentage), unless indicated otherwise.
[b] Data were adjusted for gestational age at delivery.
[c] Versus the control group.
[d] Morbidity and mortality rates are presented as a percentage of the total number of cases in each dose and treatment group.
[e] Breathing problems include respiratory distress syndrome and asphyxia.
[f] Plasma glucose levels in the newborn of less than 1.7 mmol/L within 24 h of delivery.
[g] Newborn infections that required antibiotic treatment.

below the fifth centile, but also increased mortality among the overall population, with an excess of perinatal deaths as compared with standard care [20].

Sex-dependent responses to glucocorticoid treatment have been previously found in animal and human studies with poorer outcomes reported for males [11,21,22]. Differences in placental glucocorticoid metabolizing enzymes (11-β hydroxysteroid dehydrogenase type 2; 11βHSD-2), which protect the fetus from elevated levels of maternal cortisol, could contribute to sex-dependent responses to glucocorticoids. At term, placental 11βHSD-2 activity is higher in females than in males [23], suggesting that the female fetus is exposed to lower levels of maternally derived cortisol, which facilitates autonomous development of fetal hypothalamic-pituitary-adrenal function, perhaps as an attempt to survive any further potential maternal insults [11].

The present study has some limitations. Retrospective studies are observational in nature and thus do not provide the same level of evidence as randomized controlled trials. Retrospective investigations are inherently subject to data collection bias; however, selection bias is unlikely to account for the differences observed in the present study. The healthcare workers who performed chart reviews and data collection were masked to the present study objective. Although regression analyses were used to control and adjust for possible confounding variables, including maternal parameters that did not account for the anthropometric changes after betamethasone treatment, unknown additional factors might have affected fetal weight gain and perinatal outcomes.

There are difficulties in predicting whether preterm delivery will occur within 7 days of betamethasone treatment, and further studies are required to identify and optimize predictors limiting unnecessary fetal exposure to betamethasone treatment. In the present study, 49% of males and 45% of females who had received betamethasone were born after 34^{+0} weeks. Furthermore, 28% of males and 26% of females exposed to betamethasone were born after 37^{+0} weeks and therefore might have unnecessarily received treatment. For a more selective indication, cervical length measurements on ultrasonography, vaginal fibronectin tests, and/or individualized scores to evaluate the risk of preterm delivery might become useful tools.

Although the present data provide insight into the fetal and neonatal effects of betamethasone in people, further studies are needed to determine the clinical significance of the findings and the role of the placenta in fetal growth and development after betamethasone treatment. Clinically, there should be a general awareness among practitioners for critical judgment in identifying pregnancies truly at risk of preterm delivery. The present results suggest that repetitive and high doses of betamethasone should be avoided. Sex- and gestational-age adapted betamethasone treatment for lung maturation might be considered in the future.

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Conflict of interest

The authors have no conflicts of interest.

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3 Diskussion

Die fetale Prägung beschreibt den Zusammenhang zwischen dem Einfluss exogener und endogener Faktoren in sensiblen Phasen der Fetalentwicklung und dem Zellwachstum und der Organentwicklung, letztendlich mit dem Resultat einer anhaltenden postnatalen Veränderung in Organ- und Gewebsfunktion.[1] Hierbei kann eine Fehlanpassung mit dem Auftreten von Erkrankungen im höheren Lebensalter assoziiert sein. Suboptimale intrauterine Bedingungen wie zum Beispiel maternale Unterernährung, Hypoxie, psychischer Stress oder aber die *GC-Exposition* während der Schwangerschaft können die fetale Entwicklung nachhaltig beeinflussen und sind häufig mit einer Verringerung des Geburtsgewichts assoziiert. Das mögliche Spektrum der Spätfolgen ist hierbei weit gefächert und reicht von kardiovaskulären und stoffwechselbedingten Erkrankungen bis zur Ausbildung von malignen Tumoren im Erwachsenenalter („Fetale Programming“).[14-21]

GC mit Kortisol als seinem Hauptvertreter spielen bei der Organreifung und Differenzierung in der Schwangerschaft eine wichtige Rolle.[22-24] Neben der physiologischen Erhöhung von endogenen GC zu bestimmten Entwicklungszeitpunkten können auch exogene Stressoren in der Schwangerschaft zu einer Erhöhung von endogenen GC führen und somit Einfluss auf die fetalen Programmierung nehmen und zu einer „Fehl-Progammierung“ („Mal-Programming“) führen.[15,25] Die direkte Exposition des Feten gegenüber hohen exogen GC-Spiegeln im Rahmen der LRI bei drohender Frühgeburt oder in Form einer Dauertherapie beim andrenogenitalen Syndrom („congenital adrenal hyperplasia“, CAH) hat unmittelbar klinische Relevanz.

Die hier zusammengetragenen Untersuchungen wurden mit dem Ziel durchgeführt, die kurz- und langfristigen Veränderungen der antenatalen GC-Therapie auf die Fetalentwicklung, der Entwicklung der fetalen HPA-Achse sowie dessen Interaktion mit der Plazenta, als auch die eigenständige Rolle der Plazenta für fetale Programmierung zu untersuchen. Es konnte gezeigt werden, dass sowohl die Verabreichung von BET in der Spätschwangerschaft[98] als auch die Gabe von DEX in der Frühschwangerschaft[136,324,326] zu vielfältigen Veränderungen in der Plazenta, der Fetalentwicklung, und der der HPA-Achsen Reifung führt, die sich teilweise noch postnatal nachweisen lassen.

Aus den Erkenntnissen im Tierexperiment wurden in translationalen Experimenten die dosisabhängigen Auswirkungen der antenatalen GC-Therapie mit BET auf die anthropometrischen Parameter zum Zeitpunkt der Geburt beim Menschen sowohl epidemiologisch untersucht,[116] als auch die Rolle der Plazenta, insbesondere des plazentaren Wachstumshormons Plazentalaktogen, in einer prospektiven klinischen Studie evaluiert.[123] Sowohl tierexperimentelle Untersuchungen als auch Studien am Menschen deuten darauf hin, dass insbesondere eine Exposition mit hohen antenatalen GC-Dosierungen zu geschlechtsspezifischen Veränderungen führt, deren mechanistische Grundlagen es weiter zu evaluieren gilt. Im Folgenden werden zunächst die Auswirkungen der GC-Exposition auf das fetale Wachstum und die Entwicklung der fetalen HPA-Achse diskutiert (Kapitel 3.1), anschließend dann Studienergebnisse zu plazentaren Anpassungsmechanismen (Kapitel 3.2) sowie den translationalen Ansätzen (Kapitel 3.3) diskutiert.

3.1 Auswirkungen antenataler Glukokortikoidtherapie auf die Fetalentwicklung

3.1.1 Späte GC-Exposition - Ontogeny-Study

Im Rahmen eines durch die DFG geförderten Forschungsaufenthaltes in Toronto war es möglich, in Zusammenarbeit mit den Forschungszentren in Perth, Australien und Auckland, Neuseeland die fetale Prägung im Tiermodell zu untersuchen. Im Rahmen dieses Forschungsvorhabens wurden die Auswirkungen pränataler, maternaler GC-Gaben (BET) in der Spätschwangerschaft auf das fetale Wachstum, die fetale HPA-Achse und die Plazenta beim Schaf untersucht (Ontogeny Study).[322,327]

Das gewählte Studiendesign simuliert die maternale, exogene GC-Gabe im Sinne der beim Menschen durchgeführten LRI bei drohender Frühgeburt. Hierbei wurde postuliert, dass neben der LRI die exogene GC-Exposition die fetale und postnatale HPA- und Stoffwechsel-Aktivität dosisabhängig beeinflusst und daher zu Änderungen in der Gen-Expression in der HPA-Achse und der Plazenta führt.

Trächtige Merino Schafe wurden zeitgenau gezüchtet und erhielten entweder BET in der Dosierung von 0,5mg/kg Fetalgewicht oder in der Kontrollgruppe Natriumchlorid (entsprechend dem Injektionsvolumens von BET 5-6mL). Diese Dosierung entspricht etwa der Gesamtdosis der beim Menschen verwendeten Dosis zur LRI von insgesamt 24mg BET (Celestan®). Die Muttertiere erhielten 1 (am Tag 104, n=6), 2 (am Tag 104, 111, n=6) oder 3 (am Tag 104, 111, 118, n=11) Injektionen von BET. Der Applikationszeitraum fällt in etwa in den Zeitraum der endogenen HPA-Achsenaktivierung beim Schaf und ist ähnlich dem Zeitfenster der antenatalen LRI bei drohender Frühgeburt beim Menschen (23-34 Schwangerschaftswochen). Um Erkenntnisse im Rahmen der ontogenetischen Organentwicklung zu erhalten und die Auswirkungen der GC-Gaben auf die Fetalentwicklung besser beurteilen zu können, wurden an den Tagen 75, 85, 101, 109, 116, 121, 132 und 146 die Lämmer per Sectio entbunden. Fetalgewichte und Organgewichte wurden notiert, Organproben und Plazentaproben für anschließende Untersuchungen konserviert (Kontrollgruppe n=53 Lämmer, BET behandelte Gruppe n=23). Postnatal wurden die Lämmer im Alter von 6 Wochen (n=12) und 12 Wochen (n=11) nachuntersucht. Die Auswertungen wurden bewusst nur bei Einlingsschwangerschaften durchgeführt, um eventuelle zwillingsspezifische Prägungseffekte auszuschließen.

Auswirkungen der späten maternalen BET-Gabe auf die fetale und postnatale Entwicklung

Die Arbeitsgruppe um Prof. J. Challis konnte bereits zuvor nachweisen, dass wiederholte Gaben von maternalen synthetischen GC spät in der Schwangerschaft das fetale und postnatale Wachstum reduzieren und die Entwicklung und Funktion der HPA-Achse beeinflussen.[102,137,165,242] In Untersuchungen zur BET-Gabe in der Spätschwangerschaft beim Schaf konnte eine signifikante Reduktion des Geburtsgewichtes, sowie Langzeitfolgen mit Gehirngewichtsabnahme, verminderter Myelinisierung des Nervus opticus, niedrigere IGF-1 und IGFBP-1 Plasmalevel, sowie eine Glukoseintoleranz aufgezeigt werden.[99,137,180,246,328] Des Weiteren zeigten die

Lämmer zum Zeitpunkt der Geburt als auch postnatal im Alter von einem Jahr eine verstärkte HPA-Achsenaktivität. Im Alter von drei Jahren entwickelte sich dann eine verminderte HPA-Achsenaktivität mit reduzierten basalen ACTH- und Kortisol-Plasmaspiegeln auf eine exogene Stressapplikation (CRH/AVP).[242]

Ähnlich den Angaben in der Literatur führte die BET-Exposition im Vergleich zu den Kontrolltieren in den eigenen Untersuchungen zu einer signifikanten Reduzierung des Fetalgewichts um bis zu 27%.[179,328] Der Trend der Gewichtsreduktion setzte sich postnatal Alter bis 12 Wochen fort, war dann jedoch nicht mehr signifikant (p=0,065).[322] Neben diesen Fetalgewichtsveränderungen zeigten sich auch bei den einzelnen Organgewichten wie zum Beispiel dem Gehirn, dem Cerebellum, der Hypophyse, Hippocampus, der Nebennieren, dem Herzen, der Nieren, der Leber sowie der Lunge signifikante Abnahmen in der BET Gruppe im Vergleich zu den Kontrollen und deuten auf eine erhebliche Beeinträchtigung der fetalen Organentwicklung nach BET-Therapie hin.[322] Mögliche plazentare Ursachen werden unter 3.2 erörtert.

Auswirkungen der späten maternalen BET-Gabe auf die Schlüsselgene der fetalen HPA-Achse

Li et al. untersuchten den Einfluss von BET auf die fetale HPA-Achse und die Genexpression im Hypothalamus, der Hypophyse und der Nebennierenrinde.[329] Es zeigten sich keine signifikanten Veränderungen der maternalen ACTH-Plasmaspiegel nach BET-Behandlung, aber die maternalen Kortisol-Spiegel waren am Tag 146 im Vergleich zu den Kontrollen signifikant niedriger.[382,383] Berechnet man das Verhältnis von fetalem ACTH zu fetalem Kortisol als Marker für die Nebennierenrinden-Sensitivität für fetales ACTH, so führte die BET-Behandlung zu einer signifikant abgeschwächten Nebennierenrinden Sensitivität am Tag 146.[329] Dieser Unterschied war postnatal im Alter von 6 Monaten aber nicht mehr nachweisbar. Ursächlich war eine signifikante Reduktion in der hypothalamischen mRNA-Expression von AVP im Nucleus paraventricularis.[329] Die Interaktion der fetalen HPA-Achse mit der Plazenta und die Rolle der Plazenta für die BET induzierte Programmierung der fetalen HPA-Achse wird unter 3.2 weiter ausgeführt.

3.1.2 Frühe GC-Exposition - Early DEX-Study

Im Gegensatz zu der zuvor beschriebenen „Ontogeny Study“ wurde die **„Early DEX-Study“** konzipiert, um die Auswirkungen von maternalem Stress in der Frühschwangerschaft auf die fetale und plazentare Entwicklung zu untersuchen. Der frühe Behandlungszeitpunkt am Tag 40-41 in der Schafschwangerschaft wurde gewählt, weil in diesem Zeitfenster die besonders sensiblen Phasen der plazentaren Entwicklung und Differenzierung als auch fetale endokrinologische Reifungsprozesse im Hypothalamus-Hypophysen-Portalsystem liegen.[317,330] Klinische Bedeutung hat dieser Zeitpunkt im Hinblick auf Stress in der Frühschwangerschaft, maternale Diät oder Unterernährung in der Frühschwangerschaft sowie der GC-Behandlung bei vermutetem adrenogenitalen Syndrom (CAH). Voruntersuchungen konnten zeigen, dass eine Behandlung mit DEX in der Frühschwangerschaft beim Schaf mit einer Programmierung des kardiovaskulären Systems und Hypertension im Alter von 5 Jahren einhergeht.[331]

In der hier vorgestellten Studie wurden 111 trächtige Merino-Schafe nach Randomisierung in der Frühschwangerschaft (Tag 40,41) entweder maternal mit 4x0,14mg DEX pro kg maternalem Körpergewicht über 48h (n=50) oder mit Natriumchlorid (n=61) in der Kontrollgruppe behandelt. Zwillingsschwangerschaften wurden sonographisch ausgeschlossen. An vier Untersuchungszeitpunkten in der Schwangerschaft (Tag 49-51, 101-103, 125-127 und 140-142) wurden die fetalen Lämmer per Sektio entbunden. Fetal- und Organgewichte wurden erhoben und die Gewebsproben für weitere Untersuchungen entsprechend aufbereitet. Nach Hysterektomie wurden die Plazentome einzeln aus dem Uterus entfernt, gewogen und soweit vorhanden für jedes Versuchstier jeweils separat nach Plazentomtypen (A-D) in Paraformaldehyd als auch tiefgefroren fixiert. Ein besonderes Potenzial dieser Studie liegt in der Möglichkeit der Unterscheidung zwischen männlichen und weiblichen Feten. Zur eindeutigen Identifizierung des Geschlechts in der 50 Tage alten Versuchstiergruppe bei teilweise erst rudimentär ausgebildeten sekundären Geschlechtsmerkmalen wurde eine PCR mit anschließender Gelelektrophorese von geschlechtsspezifischem pankreatischem und duodenalem homeobox 1 (PDX-1) durchgeführt.

Effekte der GC-Exposition in der Frühschwangerschaft auf die fetale und postnatale Entwicklung

Im Vergleich zu den bereits beschriebenen Fetalgewichts- und Organgewichtsveränderungen bei der GC-Exposition in der Spätschwangerschaft zeigten sich bei der Exposition in der Frühschwangerschaft deutlich geringere Veränderungen. Durch die Berücksichtigung des fetalen Geschlechts in der Early-DEX-Studie konnten hier erstmals geschlechtsspezifische Unterschiede im Fetalgewicht, der Scheitel-Steiß-Länge, im Abdomenumfang, im Gehirngewicht, im Hippocampus- und Hypophysengewicht sowie im Herzgewicht nachgewiesen werden.[332] So fanden sich zum Beispiel im Zentralnervensystem nur bei den männlichen Feten Organgewichtsveränderungen in der DEX-Gruppe im Vergleich zur Kontrollgruppe (⇑ Gehirngewicht, ⇑ Hippocampusgewicht, ⇑ Cerebellumgewicht, ⇑ Hypophysengewicht). Bei den weiblichen Feten zeigten sich hier keine Effekte der DEX-Behandlung. Allerdings war in der DEX-Gruppe das Fetalgewicht am Tag 101 im Vergleich zu den Kontrollen signifikant vermindert. Zu späteren Untersuchungszeitpunkten konnte dies nicht mehr nachgewiesen werden und lässt auf eine fetale- bzw. plazentare Kompensation vermuten (Kapitel 3.2).

Effekte der GC-Exposition in der Frühschwangerschaft auf die fetale HPA-Achse

Challis et al. konnten zeigen, dass sich die Regelkreise der fetalen HPA-Achse zwischen dem Hypothalamus und der Hypophyse beim Schaf erst nach dem Tag 50 etablieren.[162] Zu diesem frühen Zeitpunkt scheinen daher die fetalen ACTH Plasmaspiegel unabhängig von der hypothalamischen Kontrolle zu wirken. Am Tag 100 und 125 zeigte sich eine deutliche Abnahme der fetalen Kortisol-Plasmaspiegel, die gut zum triphasischen Verlauf der fetalen Nebennierenrindenaktivität während der Schwangerschaft passt: 1) Initialphase einer Nebennierenrindensensibilität gegenüber ACTH, 2) eine Phase relativer ACTH Resistenz, gefolgt von 3) einer erneuten ACTH Sensibilisierung mit vermehrter Kortisol-Ausschüttung zum Zeitpunkt der Geburt.[333-335] Paradoxerweise zeigte sich bei den Kontrolltieren, ebenso wie in der Literatur berichtet, gegen Ende der Schwangerschaft mit ansteigenden fetalen Kortisol-Spiegeln auch ein Anstieg der fetalen ACTH Spiegel, die

zunächst im Sinne der Wirkung von fetalem Kortisol im Rahmen der negativen Rückkopplung auf die fetale Hypophyse nicht plausibel erscheint.[336] Challis et al. haben postuliert, dass diese letztgenannte Aktivität während der Fetalentwicklung durch einen gleichzeitig zu beobachtenden Anstieg an CBG aus der fetalen Leber im Sinne einer positiven Vorwärts-Kopplung durch Kortisol selbst verursacht wird.[162] Diese Mechanismen sind denen des postnatalen Lebens entgegengesetzt, wenn erhöhte Kortisol-Spiegel mit einer Reduzierung der hepatischen CBG-Sekretion einhergehen, wobei die genauen Mechanismen noch unbekannt sind.[162] Ein weiterer wichtiger Mechanismus der Beeinflussung und Verstärkung der negativen Kortisol-Rückkopplung könnte eine verminderte GR Expression auf hypothalamischer-hypophysärer Ebene sein.[162]

Am Tag 50, 8-10 Tage nach Verabreichung von DEX, zeigten sich bei Feten beider Geschlechter signifikant erniedrigte Plasmakortisol-Spiegel, ohne Änderungen in den fetalen Plasma-ACTH-Spiegeln.[136] Im Gegensatz hierzu waren am Tag 140 nach DEX-Behandlung bei beiden Geschlechtern die fetalen Plasmakortisol-Spiegel signifikant erhöht. Zunächst wurde die Rolle der Hypophyse mit Hilfe von in-situ-Experimenten und Messung der mRNA-Expression von Schlüsselgenen (GR, POMC, Proconvertase Typ-1 (PC-1) und Proconvertase Type-2 (PC-2)) nach Geschlechtern und Lokalisation getrennt, untersucht. Als Untersuchungszeitpunkte standen die Tage 100, 125 und 140 zur Verfügung.

Die ontogenetischen Veränderungen in den Expressionsprofilen der gemessenen Schlüsselgene entsprechen denen aus der Literatur. Bei den Kontrollen zeigte sich die schon zuvor beschriebene lokale Verteilung der POMC-mRNA-Expression mit deutlich höherer mRNA-Expression im basalen Hypophysenanteil im Vergleich zu darüber liegenden kranialen Anteilen in der Nähe der Pars intermedia.[162] GR-mRNA ließ sich lediglich in der Pars distalis und PC-2-mRNA lediglich in der pars intermedia nachweisen. Die antenatale DEX-Behandlung führte hier nicht zu einer signifikanten Veränderung in der mRNA-Expression.[136] Das passt zu den bisherigen Annahmen, dass die POMC Expression in der Pars intermedia unabhängig von einer GC-Regulation ist und dass post-translationale Modifikationen von POMC in der Pars intermedia durch den Einfluss von CLIP (corticotropin like intermediate lobe peptide) sowie dem αMSH (alpha Melanozyten stimulierendes Hormon) stattfinden. In wieweit die Veränderungen in der GR-mRNA-Expression am Tag 100 und der POMC- und PC-1-mRNA-Expression am Tag 125 Veränderungen in der ACTH Sekretion hervorrufen, bleibt unklar. Zumindest fanden sich keine eindeutigen Korrelationen mit dem fetalen Plasma ACTH Werten. Der zuvor beschriebene Anstieg von POMC-mRNA in der Pars distalis gegen Ende der Schwangerschaft[241] ließ sich in unseren Experimenten nicht nachweisen, allerdings wurden unsere Untersuchungen bereits am Tag 140 und nicht gegen Ende der Schwangerschaft (Tag 146-150) durchgeführt.

Die Ergebnisse der fetalen Plasma ACTH Spiegel stimmen gut mit den Ergebnissen der in-situ-Experimente der Hypophyse überein. Am Tag 140 folgten die fetalen ACTH-Plasmaspiegel den in der Hypophyse gemessenen erhöhten PC-1-mRNA-Werten, und es fand sich in der DEX-Gruppe eine signifikante Erhöhung von fetalem ACTH im Vergleich zur Kontrollgruppe. Die fetalen Kortisol-Plasmaspiegel zeigten einen signifikanten

Abfall in der DEX-Gruppe im Vergleich zur Kontrollgruppe bei beiden Geschlechtern am Tag 50. Bei fehlenden ACTH Veränderungen könnte dies auf eine Nebennierenrinden-Hypersensibilität gegenüber ACTH hindeuten.

Besonders bei der Auswertung der Genexpression in der Nebenniere mit den Schlüsselenzymen und Rezeptoren der Kortisol-Synthese, ACTH-R, „steroidogenic acute regulatory protein" (StAR), P450C17 und 3ßHSD zeigten sich eindeutige Veränderungen. Hierbei entsprachen die Veränderungen im Verlauf der Schwangerschaft den bisher publizierten Ergebnissen. Bei beiden Geschlechtern fand sich ein signifikanter Anstieg der mRNA-Expression von ACTH-R, StAR, 3ßHSD und P450C17 an den Tagen 125 und 140. Es zeigte sich kein Einfluss früher maternaler DEX auf die ACTH-R-mRNA oder STAR-mRNA-Expression. Deutliche geschlechtsspezifische Unterschiede nach DEX-Behandlung konnten allerdings für die mRNA-Expression von P450C17 und 3ßSHD nachgewiesen werden. Am Tag 50 war die P450C17-mRNA Expression in der DEX-Gruppe bei den weiblichen Feten signifikant erniedrigt und mit einer Reduzierung des Kortisols im fetalen Plasma, im Sinne einer Nebennierenrinden-Hypersensibilität gegenüber ACTH, assoziiert. 100 Tage später nach DEX-Behandlung, am Tag 140 fanden sich hingegen erhöhte P450C17- und 3ßHSD-mRNA-Level bei den weiblichen Feten, die zu den gefundenen erhöhten fetalen Kortisol-Plasmawerten nach DEX-Behandlung passen. Diese Veränderungen ließen sich jedoch nicht bei den männlichen Feten nachweisen, so dass hier ein anderer Mechanismus der fetalen Kortisol-Erhöhung nach DEX-Behandlung zu Grunde liegen muss.

Die fetale hepatische CBG-mRNA-Expression war nach DEX-Behandlung nicht verändert. Die erhöhte 11ßHSD2-mRNA-Expression in der Plazenta bei den männlichen Feten würde eher den materno-fetalen Kortisol-Transfer vermindern und gibt somit ebenfalls keine Erklärung für die gefundenen Veränderungen der fetalen Plasma Kortisol-Spiegel bei den männlichen Feten. Die maternalen Plasma Kortisol-Spiegel waren im Verlauf der Schwangerschaft erwartungsgemäß unbeeinflusst von der frühen DEX-Behandlung.

Sicherlich muss man hier anmerken, dass lediglich basale mRNA-Expressionsprofile erfasst und keine Proteinbestimmungen bzw. Enzymaktivitäten gemessen wurden. Es bleibt bei der bemerkenswerten Beobachtung, dass die Aktivierung der fetalen HPA-Achse bei beiden Geschlechtern knapp 100 Tage nach einer Behandlung mit synthetischen GC in der Frühschwangerschaft verstärkt werden kann, die bei den weiblichen Feten auf einer vermehrten Aktivierung der Kortisol-Synthese in der fetalen Nebennierenrinde beruht.

Langzeitveränderungen der frühen DEX-Exposition konnten Li et al. im Alter von 30 Tagen post partum nachweisen.[337] Bei den weiblichen Lämmern zeigte sich eine verminderte Kortisol-Antwort auf einen Stressstimulus.[337] Im Alter von 7 Monaten waren die Kortisol-Plasmaspiegel in der DEX-Gruppe nach ACTH-Stresstest bei den weiblichen Feten signifikant niedriger als bei den Kontrollen.[138] Der Nachweis von reduzierte POMC- und PC-1-mRNA-Level in der Hypophyse und den daraus resultierenden niedrigeren ACTH-Plasmaspiegeln deuten, ähnlich den Ergebnissen der GC-Exposition in der Spätschwangerschaft, auf eine verminderte Reaktionsfähigkeit der Hypophyse auf Stimulation durch CRH/AVP bei den weiblichen Feten hin (Abbildung 1).[138]

In der Literatur gibt es Berichte über geschlechtsspezifische Programmierungseffekte der HPA-Achsenfunktion nach pränatalem Stress, zum Beispiel bei Ratten oder dem Meerschweinchen.[152,338] Eine kurzzeitige DEX-Behandlung bei den männlichen Feten beim Meerschweinchen führt zu einer signifikanten Erhöhung der basalen Plasma Kortisol-Spiegel, wohingegen sich bei den weiblichen Feten eine verminderte HPA-Achsenantwort zeigt. Eine länger andauernde DEX-Therapie reduziert bei männlichen Nachkommen signifikant die basalen Kortisol-Plasmaspiegel im Erwachsenenalter; bei den weiblichen Nachkommen ließen sich höhere Kortisol-Plasmaspiegel in der Follikel und Lutealphase nachweisen.[243,245] Petropoulos et al. konnten zeigen, dass die Gabe von synthetischen GC in der Schwangerschaft geschlechtsspezifisch, dosisabhängig als auch zeitpunktabhängig die Blut-Hirn-Schranke verändern können und somit alle drei Faktoren das Ausmaß der GC-Behandlung-induzierten Veränderungen der Gehirnentwicklung beeinflussen.[339]

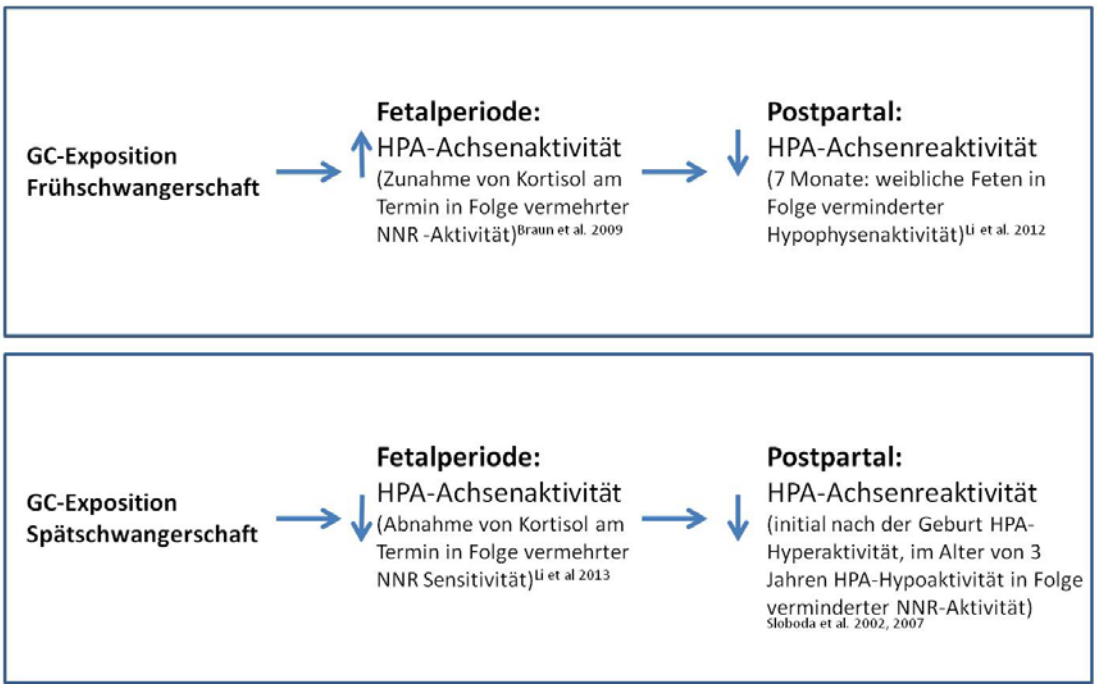

Abbildung 1: Vereinfachte Zusammenfassung der Auswirkungen der antenatalen GC-Exposition in der Früh- und Spätschwangerschaft auf die HPA-Achse.

3.2 Plazentare Anpassungsmechanismen

Prinzipiell kann man sich zahlreiche verschiedene plazentare Anpassungsmechanismen vorstellen, mit denen die Plazenta im Schwangerschaftsverlauf auf exogene Einflüsse reagiert, um eine kontinuierliche Fetalentwicklung zu gewährleisten.

Plazentagröße und Morphologie

Epidemiologische Studien zeigen eine Assoziation zwischen Plazentagewicht und dem Risiko für die Entwicklung von Krankheiten im höheren Lebensalter.[340] Größe, Gewicht als auch die Form der Plazenta variieren zum Zeitpunkt der Geburt, aber ein niedrigeres Plazentagewicht ist mit einem niedrigeren Geburtsgewicht

assoziiert, was wiederum mit einem höheren Risiko für Erkrankungen im späteren Erwachsenenalter assoziiert ist.[393,394] Findet man einen hohen Quotienten aus Plazentagewicht zu Geburtsgewicht, der auch als Marker für die „Plazentaeffizienz“ angeführt wird, so ist dieser mit einem erhöhten Risiko der Hypertension sowie koronarer Herzerkrankungen im Erwachsenenalter assoziiert.[393,395341,342] Untersuchungen zu den Veränderungen der Plazentaoberfläche und dem damit vermehrten Risiko der adulten Hypertension konnten eine Abhängigkeit von dem mütterlichen Ernährungszustand während der Schwangerschaft zeigen, wobei hier eine schlechte maternale Nährstoffversorgung ursächlich für eine kleine Plazenta angenommen wird.[343] So konnten zum Beispiel in der Helsinki Birth Cohort Study bei Männern, die zum Zeitpunkt der Geburt dünn waren, unterschiedliche materno-plazento-fetale Phänotypen in Abhängigkeit von der maternalen Größe und dem Body Mass Index (BMI) identifiziert werden. Dies stellt im Sinne der fetalen Unterernährung ein erhöhtes Risiko im Erwachsenenalter an koronarer Herzkrankheit (KHK) zu erkranken, dar.[344] Auf der anderen Seite scheint die Plazenta im Falle einer besseren maternalen Ernährungssituation durch Oberflächenvergrößerung sich dem fetalen Nährstoffverbrauch anpassen zu können. Die möglichen Mechanismen, durch die endogene GC bzw. die antenatale Behandlung mit GC einen Einfluss auf die Plazentagröße bzw. Oberfläche haben und damit den fetalen Phänotyp beeinflussen, sind weitestgehend unklar und sollten untersucht werden.

Im Gegensatz zur menschlichen hämochorialen Plazenta besitzt das Schaf eine syneptheliochoriale Plazenta und zeichnet sich durch eine nicht-invasive Plazentation aus.[315] Der zweihörnige Uterus des Schafs besitzt etwa 60-80 Karunkel, die zur Ausbildung der materno-fetalen Einheiten, den sogenannten Plazentomen, zur Verfügung stehen.[316] Historisch wurden die Plazentome gemäß ihrem morphologischem Erscheinungsbild, dem Grad der Eversion der „Hämophaguszone“, in vier Typen A-D unterteilt.[316,345] In der „Ontogeny Study“ wurden an den 8 fetalen Untersuchungszeitpunkten das Gesamtgewicht der Plazenta notiert, sowie die einzelnen Plazentome dissektiert und nach den vorbeschriebenen morphologischen Kriterien in die vier Typen, A-D eingeteilt. Im Gegensatz zur menschlichen Plazenta erreicht das Gewicht der Schafsplazenta im mittleren Schwangerschaftsdrittel ein Plateau und es folgt ein signifikanter Gewichtsabfall bis zum Ende der Schwangerschaft. Die späte maternale GC-Exposition zur weiteren signifikanten Gewichtsreduzierung des Gesamtplazentagewichts um bis zu 38% (116 und 121dG). Bei der Subgruppenanalyse für A-, B-, C- und D-Plazentome scheinen Typ-B-Plazentome mit ihrer zahlenmäßigen Zunahme und relativen Gewichtszunahme am sensibelsten auf die maternale GC-Behandlung zu reagieren.[98]

In älteren Studien wurde behauptet, es gäbe im Verlauf der Schwangerschaft eine kontinuierliche Umwandlung von Typ A- über B-, C- in Typ D-Plazentome.[317-321] In unseren eigenen Studien konnten wir zeigen, dass eine derartige kontinuierliche Umwandlung der Plazentomtypen nicht stattfindet.[135] Sowohl die Gesamtanzahl als auch das zahlenmäßige Verhältnis der Plazentome zueinander wies keine Unterschiede zwischen Tag 75 und 146 auf. Aber die maternale BET-Therapie veränderte die Zusammensetzung der Plazentom-Subty-

pen: die Anzahl der B- und C-Typen nahm ab, die Anzahl der A-Subtypen zu. Die ursprünglich auf rein morphologischen Unterschieden bestehenden Klassifikation[345] ist somit nur unzureichend, und in zukünftigen Studien muss unbedingt auf funktionale Unterschiede eingegangen werden.

Ebenfalls konnten wir in unseren Studien erste Hinweise für funktionelle Unterschiede zwischen den Plazentomtypen gewinnen.[135] Beim Schaf und anderen Wiederkäuern ist der Geburtsbeginn mit der Aktivierung der fetalen HPA-Achse und der vermehrten Sekretion von Prostaglandinen eng verknüpft. Das Schlüsselenzym der Prostaglandin-Endoperoxid-Synthase (PGTHS), welches in zwei Isoformen (PGTHS-1 und –2) vorkommt, synthetisiert Prostaglandine aus Arachidonsäure. Bei PGTHS-1 handelt es sich um die konstant exprimierte Form, bei PGTHS-2 um die induzierbare Form. Zahlreiche Substanzen, unter anderem Wachstumsfaktoren und Zytokine, nehmen Einfluss auf die Syntheseleistung. Die plazentaren PGTHS-2 Protein-Spiegel zeigten einen signifikanten, kontinuierlichen Anstieg im Verlauf der Schwangerschaft mit Maximalwerten am Ende der Schwangerschaft (Tag 146). Bezüglich der Plazentom-Subgruppenanalyse ließen sich keine Unterschiede für die PGTHS-2 Protein Expression in den Kontrollgruppen finden. Die BET-Behandlung führte allerdings am Tag 132 zu einer signifikant größeren PGTHS-2 Protein Expression in den Typ C Plazentomen im Vergleich zu den Typ A Plazentomen.[135] Vermutlich besitzen insbesondere die B- und C-Plazentome kompensatorische Fähigkeiten um auf Veränderungen des intrauterinen Milieus zu reagieren und damit die fetale Wachstumskapazität aufrecht zu erhalten.

Im Gegensatz zu den Veränderungen in der Plazentom-Verteilung nach BET-Gabe in der Spätschwangerschaft (Zunahme der A-Plazentome mit signifikante Abnahme der B- und C-Plazentome am Tag 125)[136] konnten wir nach DEX-Gabe in der Frühschwangerschaft nur dezente Veränderungen nachweisen. Weder die Gesamtzahl der Plazentome noch das Gesamtplazentagewicht waren nach DEX-Therapie verändert.[324] Nur bei den männlichen Feten konnte nach DEX-Therapie ein Plazentomshift von A- zu den mehr „metabolisch aktiven“ B- und C-Plazentomen beobachtet werden. Bei den weiblichen Feten ließ sich dies nur für die C-Plazentome zeigen.[324] Wir vermuten daher, dass das fetale Wachstumspotenzial bei den männlichen Feten trotz der frühen DEX-Therapie durch einen Plazentomshift zu den B- und C-Plazentomen aufrechterhalten werden konnte.

Endokrinologische Veränderungen - Plazentalaktogen

Im Hinblick auf die funktionellen Veränderungen in der Plazenta und dem Fetalgewichtsreduktion nach maternaler GC-Exposition wurde das plazentare Laktogen (PL), ein Wachstumshormon in der Plazenta, untersucht. PL nimmt mammo-, lakto- und sommatotrop Einfluss auf den maternalen und fetalen Stoffwechsel. Das PL beim Schaf (oPL) wird von sogenannten binukleären Zellen (BNC), die aus dem fetalen Synzytiotrophoblasten stammen, sowohl in die maternale als auch die fetale Blutzirkulation sezerniert. Die binukleären, fetalen Trophoblastzellen (BNC) stellen 20-30% des fetalen Trophoblasten.[346] Durch Verwendung des für das Schaf spezifischen Antikörpers war es möglich, die binukleären Zellen, die oPL produzieren, in der Schafsplazenta zu untersuchen.

Die maternale BET-Exposition in der Spätschwangerschaft führte zu einer ausgeprägten Reduzierung in der Anzahl der BNC von bis zu 47%. Diese Reduzierung hielt auch nach Abschluss der maternalen BET-Exposition bis zum Ende der Schwangerschaft an. Untersuchungen zur Lokalisation und Verteilung von BNC innerhalb eines Plazentoms wurden entsprechend drei Leveln vorgenommen, die in etwa den zuvor von Burton et al. beschriebenen drei Zonen im Plazentom entsprechen.[347]

Ältere Studien zeigen, dass BNC nach Wanderung von der fetalen zur maternalen Seite und Fusion mit dem feto-maternalen Syncytium, oPL in die maternale Zirkulation abgeben.[348] Es wird vermutet, dass intrafetale Kortisol-Gaben hierbei entweder Einfluss auf den Migrationsprozess oder aber auf die Neubildung von BNC haben und diese hemmen.[349] Unsere Untersuchungen lassen vermuten, dass die antenatale BET-Behandlung den natürlich vorkommenden Anstieg/Neubildung von BNC zwischen Tag 75 und Tag 109 inhibiert. Wir konnten ebenfalls zum ersten Mal zeigen, dass die antenatale BET-Behandlung das Verteilungsmuster von BNC innerhalb eines Plazentoms beeinflusst, was die Auswirkungen für die Plazentafunktion haben könnten.
Es ist seit langem bekannt, dass der plazentare Nährstofftransport maßgeblich von der Gefäßentwicklung in der Plazenta abhängt. Die plazentare Gefäßentwicklung beim Schaf findet vor allem durch eine Zunahme der Anzahl als auch der Flächendichte von Kapillaren insbesondere im fetalseitigen Plazentom statt.[350,351] Die plazentare Austauschfläche für den Nährstofftransport nimmt vor allem durch die Längenzunahme und vermehrte Gefäßaussprossung zu. [350,352] Beim Schaf wandern die BNC aus dem fetalen chorialen Epithel durch Tight Junctions im fetalen Trophoektoderm, um mit dem Syncytium zu verschmelzen.[353] Der Nachweis von BNC in allen drei Level lässt vermuten, dass diese sich kontinuierlich über Fusion aus zwei einzellkernigen Zellen ohne anschließende Reduktion des Zellkernmaterials[354-356] über die gesamte Schwangerschaft parallel zur Zottenausbildung entwickeln.

Neben der Abnahme der Anzahl der BNC zeigte sich auch für das oPL-Protein in der BET-Gruppe eine signifikante Reduktion an den Tagen 116 und 121 von bis zu 72%. Sowohl im maternalen als auch im fetalen Plasma fanden sich entsprechend den Befunden zu der Anzahl der BNC und den oPL-Protein Konzentrationen signifikante Abfälle im Plasma oPL nach maternaler BET-Behandlung an den Tagen 109, 116 und 121 (bis zu 78% im maternalen und bis zu 63% im fetalen Plasma). Der parallel verlaufende Anstieg in der oPL-Protein und oPL-Plasmakonzentration ab dem Tag 132 deutet auf eine vermehrte BNC-Produktion bei gleichbleibend reduzierter BNC-Anzahl hin. Dies könnte als funktioneller Anpassungsmechanismus der BNC nach antenataler BET-Behandlung angesehen werden.
Maternale oPL-Plasmakonzentrationen und Fetalgewicht korrelierten positiv. Dieser Zusammenhang konnte nicht für fetale oPL-Plasmakonzentrationen gefunden werden. Hiernach scheint insbesondere dem maternalen oPL in der Spätschwangerschaft eine besondere Bedeutung für die Fetalgewichtsentwicklung zuzukommen.

Zusammenfassend konnten wir somit zeigen, dass bereits nach einmaliger Gabe BET in klinisch relevanter Dosis die Anzahl der BNC im Vergleich zu den Kontrollplazenten signifikant und anhaltend verringert war.[98] Die Reduktion der BNC war mit einer Verringerung der oPL-Proteinlevel in der Plazenta sowie mit einer Verringerung der oPL-Hormonlevel im maternalen und fetalen Plasma assoziiert.[98] Reduzierte oPL-Hormonspiegel könnten somit direkt oder über die Regulation assoziierter Stoffwechselwege zu der mit BET-Gaben einhergehenden Geburtsgewichtsverringerung führen. Die deutlichen Auswirkungen von maternalen GC auf die BNC und damit einhergehenden Reduktion des plazentaren Wachstumshormons könnten eine Erklärung für die Reduzierung des Fetalgewichts nach BET-Behandlung.

Auch nach früher maternale DEX-Therapie zeigten sich signifikante Auswirkungen auf die Anzahl der BNC und deren oPL-Produktion, allerdings nur bei den weiblichen Feten.[324] So führte die frühe maternale DEX-Therapie lediglich am Tag 100 zu einer signifikanten Reduktion der Anzahl der BNC im Vergleich zu den Kontrollen, insbesondere bei den A-Plazentomen. Bei der Analyse der oPL-Protein Konzentrationen war es uns jetzt erstmals möglich, neben der bislang stets erfolgten Gesamtbanden-Analyse (Doppelbande), dank neuster Bildanalyse Verfahren (ChemiDoc™ MP System, Bio-Rad Laboratories GmbH München, Deutschland) und weiter optimierten Western Blot Protokoll, die oPL-Banden einzeln zu untersuchen.[324]

So konnten wir zum ersten Mal einen Einfluss der frühen maternalen DEX-Therapie auf die Glykosylierungsrate von oPL nachweisen.[324] Interessanterweise zeigten sich bei den fetalen und maternalen oPL-Plasmaspiegeln keine Veränderungen nach früher DEX-Therapie. Diese Daten lassen vermuten, dass eine differenzielle oPL-Abgabe der BNC an die maternale (Wanderung und Exozytose) und fetale (Sekretion) Zirkulation vorliegt und diese durch die DEX-Therapie in unterschiedlicher Weise beeinflusst wird.

GC-Exposition – Induktion plazentarer Apoptose

Sowohl der Anstieg von endogenem Kortisol gegen Ende der Schwangerschaft, als auch durch die exogene maternale GC-Therapie in der Früh- und Spätschwangerschaft reduziert sich die Anzahl der BNC.[24,98,162,357,358] Bislang war unklar, ob diese zahlenmäßige Reduktion der BNC durch eine Verminderung der BNC-Formation, einer vermehrten Migration und/oder durch Apoptose hervorgerufen wird.[98]

In unseren Experimenten zur frühen DEX-Exposition konnten wir zeigen, dass die geschlechtsspezifische Reduktion in der Gesamt-Zellzahl der BNC nach DEX-Therapie von einer Induktion von plazentaren Apoptose-Markern begleitet war (↑ Caspase-3-, ↓ PCNA-mRNA-Level).[57] Am Tag 125 bestanden keine signifikanten Unterschiede mehr im Fetalwachstum bei den weiblichen Feten. Die die Gesamtzahl der BNC in der DEX-Gruppe glich der Kontrollgruppe. Gegen Ende der Schwangerschaft ließen sich keine veränderten pro- oder anti-Apoptose Marker mehr nachweisen.[57] Die bei den männlichen Feten gefundenen Veränderungen in der Plazenta waren hierbei unabhängig von den untersuchten plazentaren Apoptose-Markern.

Glukokortikoidrezeptor – Möglichkeit der geschlechtsspezifischen Programmierung

Die Wirkung der GC im Gewebe wird durch GC-Rezeptoren (GR) vermittelt, die im Zellkern als ligandenabhängige Transkriptionsfaktoren wirken.[211] Es war daher wichtig, im Zusammenhang mit den Auswirkungen der GC-Therapie auf die Plazenta, ebenfalls die GR-Expression in der Plazenta zu untersuchen. Im Menschen konnten bislang zwei GR-Isoformen bestimmt werden, die von einem einzelnen mRNA-Transkript abstammen.[359] Die ligandenabhängige GR_{α} Isoform stimuliert im Zielgewebe die Transkription und wird als aktive Form beschrieben.[220] Bei der ligandenunabhängigen GR_{β} Isoform vermutet man eine hemmende Wirkung auf den GRα.[223]

Im Schafsmodell kommt es gegen Ende der Schwangerschaft mit dem natürlichen Anstieg des fetalen Kortisols als Vorbereitung auf die Geburt zu einem Anstieg der GR_{Total}- und GR_{α}-Protein-Spiegel, $GR_{ß}$-Protein-Spiegel zeigten hingegen keine Veränderungen.[360] Mittels Immunhistochemie wurden die Plazentome auf die Verteilung von GRt und GRα untersucht. Hierbei konnten wir erstmalig zeigen, das GRα neben der üblichen uninukleären Lokalisation sich ganz spezifisch in den Zellkernen der BNC darstellen lässt.[361] Unterschiedliche Anfärbemuster von BNCs mit GR_{α} lassen vermuten, dass unterschiedliche Aktivierungs-/Reifezustände von BNC existieren. Diese sind nach Leveln und Schwangerschaftstagen unterschiedlich verteilt. Während es zu einem signifikanten Anstieg der „aktiven" BNC Form zwischen Tag 50 und 100 kommt, nehmen die „inaktiven" und „intermediären" BNC Formen in diesem Zeitraum ab. Zum Zeitpunkt der Geburt zeigt sich ein umgekehrtes Bild, die Anzahl der „aktiven" BNC nimmt wieder ab, die Anzahl der „inaktiven" und „intermediären" BNC Formen wieder zu. DEX in der Frühschwangerschaft unterbricht diese Entwicklung, das heißt die Anzahl der aktiven BNC blieb konstant erhöht und könnte somit ein Hinweis für einen plazentaren Kompensationsmechanismus sein, das fetale Wachstumspotenzial trotz früher DEX-Behandlung aufrecht zu erhalten.[326,362]

„Aktive" GRα-Zellen korrelieren positiv mit den maternalen und fetalen oPL-Spiegeln und zeigten eine positive Korrelation zwischen der Anzahl positiv gefärbter GRα BNC und den maternalen und fetalen oPL-Plasma-Spiegeln sowie dem Fetalgewicht. Es war uns somit erstmalig möglich, nach der spezifischen intranukleären GRα-Lokalisation in BNC, drei GRα-BNC-Phänotypen und funktionelle Unterschiede durch Doppelfärbungen immunhistochemisch nachzuweisen.**[326,361]** Die maternale DEX-Therapie führte hierbei zu Veränderungen in der Verteilung der drei GRα-BNC-Phänotypen mit Zunahme der „aktiven" und Abnahme der „inaktiven" BNC-Form.**[326,362]**

Mortalität und Morbidität weiblicher und männlicher Feten unterscheiden sich zu Ungunsten männlicher Feten durch Komplikationen wie plazentarer Insuffizienz, IUGR, Präeklampsie, Frühgeburt und Infektionen.[63,183,363-367] Die physiologischen Mechanismen hinter diesen geschlechtsspezifischen Unterschieden sind bislang nur wenig erforscht. Bereits in der normalen Schwangerschaft lassen sich geschlechtsspezifische Unterschiede im plazentaren GC-Metabolismus (11ßHSD2-Aktivität) nachweisen; die 11ßHSD2-Aktivität in Plazenten von weiblichen Feten ist deutlicher größer als bei den männlichen Feten.[368] Man vermutet, dass bei

weiblichen Feten die intrazellulären Kortisol-Spiegel im Hinblick auf die Sensitivität bzw. als zelluläre Antwort auf eine GC-Exposition deutlich niedriger sind.[64] Die maternale Asthmatherapie mit GC bei schwangeren Frauen führte lediglich bei den weiblichen Feten zu einer Reduktion des Fetalgewichts bei gleichzeitiger Zunahme der plazentaren 11ßHSD2-Aktivität.[63] Bei den männlichen Feten ließen sich weder Gewichtsveränderungen noch Änderungen in der plazentaren 11ßHSD2-Enzymaktivität nachweisen.[63] Man vermutet daher, dass der weibliche Fet durch Anpassung der plazentaren Funktion und der damit einhergehenden Reduktion des fetalen Wachstums für eine maternalen Erkrankungen/Exposition kompensiert. Das Ausbleiben dieses Kompensationsmechanismus (fehlender 11ßHSD2-Anstieg, vermehrte intrazelluläre Sensitivität gegenüber Kortisol und vermehrte Immunantwort bei den weiblichen Feten) scheint bei den männlichen Feten zu einer erhöhten Morbidität und Mortalität zu führen.[63,369] Inwieweit diese Unterschiede in der plazentaren Enzymaktivität auch für geschlechtsspezifische Unterschiede im Langzeitüberleben verantwortlich sind, bleibt noch zu klären.

Auch in unserem Tiermodell fanden sich zahlreiche geschlechtsspezifische Unterschiede bei der plazentaren und fetalen Entwicklung nach früher maternaler DEX-Therapie. Geschlechtsspezifische Veränderungen in den fetalen, aber nicht maternalen Plasma Insulin und Glukosespiegeln deuten auf eine DEX-induzierte Insulinresistenz hin.[370] Des Weiteren führte die maternale DEX-Therapie zu einer geschlechtsspezifischen vermehrten Expression von einigen Schlüsselenzymen der Kortisol-Synthese in der fetalen Nebennierenrinde zum Zeitpunkt der Geburt, die in einer vermehrten fetalen Kortisol-Synthese bei den weiblichen Feten resultierte (Kapitel 3.1.2).[136] Neben der veränderten HPA-Achsenaktivität[136] und der postnatalen endokrinen Reagibilität[73,148,152,153] zeigten weibliche Feten transiente plazentare Anpassungsmechanismen in Bezug auf die Plazentomverteilung und Funktion.[324] Die maternale DEX-Therapie in der Frühschwangerschaft verursachte bei den weiblichen Feten eine transienten Geburtsgewichtsveränderung, die mit einer verminderten Anzahl an BNC einhergeht und eine vermehrte plazentare Apoptose induziert, ohne dass sich jedoch langfristige Veränderungen in den plazentaren oPL-Protein-Spiegeln nachweisen ließen.[324] Die weiblichen Feten zeigten ein vermindertes Wachstum, das man im weitesten Sinne als einen Anpassungsmechanismus werten kann, um weitere maternale Insulte zu überstehen.[146,154]

In einer erst kürzlich veröffentlichten Studie konnten in der humanen Plazenta neben den bekannten GR-Isoformen GRα und GRß insgesamt 10 weitere GR Isoformen identifiziert werden.[371] Variationen in der GR-Isoform Expression könnten eine besondere Rolle in der GC-Sensitivität der Plazenta spielen. Die selektive Expression der GRα-D Isoform in unreifen dendritischen Zellen der Maus zum Beispiel führte zu einer vermehrten GC-Resistenz.[163,164] Im Gegensatz hierzu bewirkte eine vermehrte Expression von GRα eine vermehrte GC-Sensibilität. Verschiedene GR-Isoformen könnten zu der gewebsspezifischen GC-Sensibilität bei der ontogenetischen Reifung und Regulation von Organen beitragen, letztendlich mit dem Resultat einer geänderten GC-Resistenz oder GC-Sensibilität in der Schwangerschaft. Hierbei involviert sind das Enzym 11ß hydroxysteroid dehydrogenase type 2 (11ßHSD2) als transplazentare Kortisol-Schranke,[372] das Glykoprotein

P (PgP)[373] oder das Kortisol bindende Globulin („cortisol binding globulin“, CBG),[374] die die Höhe der Kortisol-Exposition an der Plazenta und dem Feten modulieren. Dennoch lassen sich die beobachteten geschlechtsspezifischen feto-plazentaren Reaktionen auf eine Kortisol-Exposition durch diese Mechanismen der prä- und post-GR Modulation hierdurch nicht vollständig erklären.[375-377]

Man vermutet, dass weibliche Feten sich an eine hohe Kortisol-Exposition durch Modulation ihrer GC-Stoffwechselwege anpassen und dies mit einem verminderten fetalen Wachstum einhergeht. Durch diese Anpassung könnte es den weiblichen Feten im weiteren Verlauf der Schwangerschaft möglich sein, weitere intrauterine Insulte mit Verminderung zum Beispiel der Nahrungszufuhr oder von Sauerstoff zu überstehen und die für die Spezies bedingte Reproduktionsfähigkeit durch ein intrauterines Überleben des Feten damit gesichert ist.[377] Studien an Schwangeren mit Asthma konnten zeigen, dass die GR-Isoform-Expression mit dem Zelltyp, der zellulären Lokalisation, dem Ausmaß an Wachstumsrestriktion und dem fetalen Geschlecht variiert.[371] Männliche Feten könnten eine gewisse GC-Resistenz vor allem durch die hemmenden Eigenschaften des GRß in einer intrauterinen Umgebung mit hohen Kortisol-Werten aufweisen.[371] Weibliche Feten scheinen eher eine GC-Sensibilität durch die Ko-Expression von GRα-A mit GRα-C und GRα-D3 aufrecht zu erhalten.[371]
Interessanterweise fand sich bei unseren eigenen Untersuchungen zu den Auswirkungen der frühen maternalen DEX-Therapie am Schaf eine signifikante Abnahme in den GRt-Protein-Spiegeln, die sich jedoch nicht bei den GRα-Protein-Spiegeln nachweisen ließen. Diese Befunde legen ebenfalls nahe, dass auch hier weitere GR-Isoformen existieren und diese für die geschlechtsspezifischen Unterschiede in der GC-Sensitivität verantwortlich gemacht werden könnten.

3.3 Übertragbarkeit auf klinische Studien

In einer großen, retrospektiven Fallkontrollstudie an über 44.000 Schwangerschaften haben wir die dosis- und geschlechtsspezifischen Effekte einer antenatalen LRI mit BET bei drohender Frühgeburt auf die fetale Anthropometrie und das neonatale Outcome beim Menschen untersucht.[116] Hierbei konnten wir zeigen, dass bereits eine Einmalgabe von 2x12mg BET im Mittel das Geburtsgewicht um 154g reduziert. Die wachstumsreduzierenden Eigenschaften von BET ließen sich in dieser Studie auch erstmalig in antenatalen Ultraschallfolgeuntersuchen nachweisen.

Generell war das neonatale Outcome nach BET überraschenderweise nicht besser. Lediglich bei den männlichen Feten, die als Frühgeborene vor 37+0 SSW geboren wurden, konnte eine signifikante Verbesserung des neonatalen Outcomes nach BET im Vergleich zu den Kontrollen nachgewiesen werden. Feten profitierten nicht von einer BET-Behandlung zwischen 23+5 und 34+0 SSW, wenn sie nach 37+0 SSW geboren wurden. Hier waren die Apgar-Werte in der BET-Gruppe sowohl bei den weiblichen als auch bei den männlichen Feten

im Vergleich zu den Kontrollen signifikant schlechter. Es bestand eine BET-Dosisabhängigkeit, wobei eine höhere BET-Dosierung mit einer stärkeren Verringerung des Wachstums verbunden war, aber nicht mit einer verbesserten neonatalen Morbidität oder Mortalität: Im Gegenteil, höhere Dosierungen waren bei den männlichen Feten mit einer signifikanten Verschlechterung der Apgar-Werte assoziiert.[378]

Diese geschlechtsspezifischen Unterschiede ließen sich ähnlich auch in einer weiteren Studien zu den Auswirkungen antenataler BET-Therapie bei Zwillingsschwangerschaften aufzeigen. Unterschiede in der Pharmakokinetik von BET zwischen Einlingsschwangerschaften und Mehrlingen mit einer erhöhten BET-Clearance bei Zwillingen im Vergleich zu den Einlingen[379] ließen einige Autoren spekulieren, dass die üblicherweise bei den Einlingen empfohlenen BET/DEX-Dosierungen für Mehrlinge zu niedrig dosiert sein könnten.[87,380-382] In einer retrospektiven Fallkontrollstudie an 1922 Zwillingsschwangerschaften wurden die Auswirkungen antenataler LRI mit BET (n=653) auf das fetale Wachstum und neonatale Outcome im Vergleich zu nicht behandelten Zwillingsschwangerschaften (n=1269) untersucht.[378] Mit Hilfe eines Zwei-Stufen Zwillingsmodells (Gemischt-lineares Modell) war es uns möglich, die BET-Effekte unabhängig von den sonstigen Umwelteinflüssen zu untersuchen. Auch in dieser Untersuchung verminderte die antenatale BET-Behandlung das Geburtsgewicht (-5.3%), die Körperlänge (-1.7%) und den Kopfumfang (-2.7%) signifikant und unabhängig von einer Vielzahl von Confoundern. Geschlechtsspezifische Unterschiede konnten auch hier gezeigt werden. Die Geburtsgewichtsverminderung war deutlich größer bei den weiblichen und gemischt-geschlechtlichen Zwillingspaaren im Vergleich zu den männlichen Zwillingspaaren im Mittel bei den weiblichen Paaren -134g, gemischt geschlechtlichen Paaren -119g und den männlichen Paaren -95g im Vergleich zu den jeweiligen alters- und Paarstruktur gematchten Kontrollen.[378] Höhere Dosierungen führten bei den weiblichen Feten zu einer stärkeren Geburtsgewichtsreduktion ((≤16 mg –114 g; 24 mg –124 g; >24 mg –187 g), ohne dass diese Dosissteigerung mit einer Verbesserung des neonatalen Outcomes einherging.[378] Die Raten an Atmungsproblemen, neonataler Hyperbilirubinämie und neonatalen Infektionen war bei Feten mit matenaler BET-Therapie >24mg ähnlich denen der Kontrollen ohne BET-Behandlung. Bei den gemischt-geschlechtlichen Paaren zeigte sich nur bei den weiblichen Feten eine Reduktion des Geburtsgewichts im Vergleich zu altersgepaarten Kontrollen. Diese Untersuchungen an Zwillingen mit gleichem genetischen Hintergrund legen nahe, dass die geschlechtsspezifisch unterschiedliche Beeinträchtigung des fetalen Wachstums durch die antenatale BET-Therapie nicht nur durch den Einfluss der Genetik erklärbar ist, sondern den Geschlechtshormonen für die GC-Sensibilität und die Beeinträchtigung der Fetalentwicklung eine eigene Rolle zukommen. Dies bietet Potenzial für zahlreiche weitere Untersuchungen.

Eine direkte klinische Relevanz aus den Ergebnissen der Einlingsstudie[116] als auch aus den Daten der Zwillingsstudie[378] konnte ebenfalls abgeleitet werden. Bei den Einlings-Schwangeren blieben 70% mehr als 7 Tage nach initialer BET-Behandlung unentbunden, 48% wurden nach 34+0 SSW entbunden.[116] Bei den Mehrlingen blieben 74% der mit BET behandelten Frauen länger als sieben Tage unentbunden, 46% wurden nach 34+0 SSW entbunden.[378] Diese Zahlen machen unter anderem deutlich, wie schwierig es ist, den Verlauf einer

drohenden Frühgeburt abzuschätzen und nur für diejenigen eine Indikation zur LRI mit GC zu stellen, bei denen tatsächlich eine Frühgeburt innerhalb der kommenden 7-19 Tage und vor 34+0 SSW stattfindet. Kombinierte Untersuchungen mit Einbeziehung der Anamnese, der klinischen Beschwerden, der transvaginalen sonographischen Zervixlängenmessung sowie der quantitativen Bestimmung von fetalem Fibronektin könnten hierbei individualisiert, eine bessere Abschätzung der Notwendigkeit der antenatalen LRI mit GC bei drohender Frühgeburt bieten.[58]

Entsprechend den in unseren tierexperimentellen Studien am Schaf bislang gewonnen Ergebnissen zu den Auswirkungen antenataler GC-Therapie auf die Fetalentwicklung und den Veränderungen in der Plazenta haben wir einer prospektiven Studie beim Menschen die Auswirkungen einer Einmalgabe BET (2x12mg n=44, Kontrollen n=49) auf das fetale Wachstum und die Plazenta untersucht.[123]

Bereits eine Einmalgabe von 2x12mg BET verminderte das Geburtsgewicht (-18.2%), den Kopfumfang (-8.6%), die Körperlänge (-6.0%) und die Plazentagröße (-5.5% Breitenabnahme).[123]

Wie im Tierexperiment gezeigt, führte auch beim Menschen die antenatale BET-Therapie zu histomorphologischen Veränderungen in der humanen Plazenta mit Zunahme des Kernumfangs und Fläche des Synzytiotrophoblasten als Syntheseort des humanen Plazentalaktogens.[123] Einen direkten kausalen Zusammenhang zwischen der Geburtsgewichtsreduktion und der Rolle von Plazentalaktogen, wie im Schafsexperiment eindrücklich demonstriert, konnten wir jedoch im humanen Modell nicht zeigen. Hier scheinen andere Regulationsmechanismen eine Rolle zu spielen.

4 Zusammenfassung

Epidemiologische und experimentelle Studien haben einen wichtigen Zusammenhang zwischen intrauteriner Wachstumsrestriktion, niedrigem Plazenta- und Geburtsgewicht einerseits und der fetalen Prägung von Erkrankungen im späteren Leben andererseits gezeigt.

In den Untersuchungen am Schaf zur maternalen GC-Behandlung sowohl in der Früh- als auch in der Spätschwangerschaft konnten eine Reduktion des Geburtsgewichts sowie Veränderungen in den Organgewichten nachgewiesen werden. Auch funktionelle Veränderungen mit einer veränderten HPA-Achensaktivität sowohl in der Fetalperiode als auch postnatal konnten nach maternaler GC-Behandlung gezeigt werden.

Eine mögliche Rolle für die GC-vermittelten Prägungseffekte kommt hierbei der Plazenta zu. Die Auswirkungen von maternalen GC auf die BNC beim Schaf und eine damit einhergehenden Reduktion des plazentaren

Wachstumshormons könnten eine Erklärung für die Reduzierung des Fetalgewichts nach BET-Behandlung sein, entweder direkt oder über die Regulation assoziierter Stoffwechselwege.

In Untersuchungen zu den potenziellen Regulationsmechanismen der BNC via GR konnten wir erstmals drei unterschiedliche Aktivitäts- bzw. Reifungsformen von BNC nachweisen. Die GC-Behandlung führte zu einem veränderten Verteilungsmuster mit einer Reduktion der Anzahl an „inaktiven" BNC und einem Shift zu den „aktiven" BNC.

Von besonderer Bedeutung sind hierbei unsere Beobachtungen zu den geschlechtsspezifischen Anpassungsstrategien an das intrauterin veränderte Milieu nach GC-Therapie. Unsere Daten zu den GR-Proteinleveln (GRt, GRα) lassen ähnlich den kürzlich publizierten Daten in der humanen Plazenta das Vorhandensein noch weiterer GR-Isoformen vermuten, die für die geschlechtsspezifischen Unterschiede und GC-Sensibilität verantwortlich sein könnten. Während bei weiblichen Feten offenbar eine konstante plazentare GC-Empfindlichkeit aufrechterhalten wird (konstante GRt-Spiegel), möglicherweise im Sinne einer präferenziellen Überlebensstrategie zur Sicherung der Reproduktionsfähigkeit und Arterhaltung, scheint es bei männlichen Feten infolge erhöhter GC-Exposition zu einem mindestens temporären Zustand erworbener plazentarer GC-Resistenz zu kommen (verminderte GRt-Spiegel am Tag 125). Wir vermuten, dass die geschlechtsspezifische Sensibilität gegenüber GC durch eine unterschiedliche Verteilung, Expression und/oder Interaktion von plazentaren GR-Isoformen bedingt ist und die maternale GC-Exposition diese nachhaltig beeinflusst.

Übertragen auf den Menschen wurden sowohl in retrospektiven Fallkontrollstudien bei Einlings- und Mehrlingsschwangerschaften als auch in einer prospektiven Studie die Auswirkungen der maternalen antenatalen GC-Exposition beim Menschen bei drohender Frühgeburt untersucht. Auch in diesen Studien konnten wir wachstumsvermindernde Eigenschaften antenataler BET-Therapie sowohl vorgeburtlich durch pränatalen Ultraschall als auch zum Zeitpunkt der Geburt nachweisen. Eine eindrückliche BET-Dosisabhängigkeit konnte gezeigt werden, wobei eine höhere BET-Dosierung mit einer stärkeren Verringerung des Wachstums verbunden war, nicht jedoch wie bislang vereinzelt vermutet, mit einer verbesserten neonatalen Morbidität oder Mortalität einherging. Untersuchungen an Zwillingen zeigten, dass die geschlechtsspezifisch unterschiedliche Beeinträchtigung des fetalen Wachstums durch die antenatale GC-Therapie nicht nur durch den Einfluss der Genetik erklärbar ist, sondern hier der Rolle von Geschlechtshormonen für die GC-Sensibilität im Sinne einer hormonalen Prägung eine besondere Rolle zukommen dürfte.

Der Plazenta kommt hierbei natürlich eine besondere Bedeutung zu. Unsere Untersuchungen lassen vermuten, dass die Plazenta, mehr als bisher angenommen eine wichtige Rolle im Zusammenhang mit der fetalen Prägung spielt. Die morphologischen und strukturellen Eigenschaften der Plazenta, die Plazentagröße, die Blutversorgung, das Vorhandensein von Transportermolekülen und die Fähigkeit der Plazenta, Nährstoffe selbst zu verstoffwechseln und Hormone zu produzieren, sind hier von besonderer Bedeutung. Im Gegensatz

zum Tierexperiment konnte in unseren Studien am Menschen ein direkter kausaler Zusammenhang zwischen GC-induzierter fetaler Gewichtsreduktion und dem humanen plazentaren Wachstumshormon Plazentalaktogen nicht nachgewiesen werden.

5 Ausblick

Die antenatale Behandlung mit GC scheint mit einer geschlechtsspezifischen fetalen Wachstumsrestriktion sowie strukturellen und funktionellen Veränderungen in der Plazenta assoziiert zu sein, die potenziell die Gesundheit im späteren Leben beeinflussen können. Sowohl im Tiermodell als auch beim Menschen sind geschlechtsspezifische Anpassungsstrategien an ein verändertes intrauterines Milieu beschrieben. Während bei den weiblichen Feten eine eher kontinuierliche Sensibilität gegenüber GC zu bestehen scheint, möglicherweise im Sinne einer präferentiellen Überlebensstrategie zur Sicherung der Reproduktionsfähigkeit mit Spezieserhalt, scheint bei männlichen Feten nach GC-Exposition die Plazenta zumindest temporär eine Resistenz gegenüber GC zu entwickeln. Unser Verständnis der Wirkung von endogenen GC und/oder Überexposition mit exogenen GC auf die Fetal- und Plazentaentwicklung beginnt maßgeblich mit dem GR und seinen Isoformen. Die Entschlüsselung der geschlechtsspezifischen Anpassungsmechanismen im Rahmen antenataler GC-Behandlung und/oder Stressexposition könnte dazu beitragen, selektive GR-Modulatoren zu entwickeln. Wir vermuten, dass die geschlechtsspezifische Sensibilität gegenüber GC durch ein unterschiedliches GR-Verteilungsmuster, Expression und/oder Interaktion des stimulierenden GRα- vs. anderen GR-Isoformen verursacht wird, und dass die Behandlung mit BET dieses beeinflusst. Zellspezifische Unterschiede in der GR-Isoform-Expression könnten nachgeschaltete Signalwege, wie die Kortisol-Plazentaschranke (11ßHSD2) oder den plazentaren Glukosetransport, die durch Kortisol reguliert werden, beeinflussen. Hierauf orientierte Studien dürften die Identifizierung von Biomarkern sowie potenzielle Angriffspunkte bzw. Optimierungen zur Behandlung der Frühgeburt in späteren Studien ermöglichen. Ein verbessertes Verständnis für die Plazenta-vermittelten Signalwege, die zur fetalen Programmierung beitragen, werden von entscheidender Bedeutung bei den Bemühungen sein, interventionelle Strategien für Risikogruppen zu entwickeln.

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7 Danksagung

In der Online-Ausgabe dieser Habilitationsschrift ist aus Gründen des Datenschutzes die Danksagung nicht enthalten.

8 Eidesstattliche Erklärung

Erklärung

§ 4 Abs. 3 (k) der HabOMed der Charité

Hiermit erkläre ich, dass

- weder früher noch gleichzeitig ein Habilitationsverfahren durchgeführt oder angemeldet wurde,
- die vorgelegte Habilitationsschrift ohne fremde Hilfe verfasst, die beschriebenen Ergebnisse selbst gewonnen sowie die verwendeten Hilfsmittel, die Zusammenarbeit mit anderen Wissenschaftlern/ Wissenschaftlerinnen und mit technischen Hilfskräften sowie die verwendete Literatur vollständig in der Habilitationsschrift angegeben wurden,
- mir die geltende Habilitationsordnung bekannt ist.

Ich erkläre ferner, dass mir die Satzung der Charité – Universitätsmedizin Berlin zur Sicherung Guter Wissenschaftlicher Praxis bekannt ist und ich mich zur Einhaltung dieser Satzung verpflichte.